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Review Article

PHARMACOMETABOLOMICS: ASPECTS AND PROSPECTS

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ABSTRACT

Metabolomics, a novel "omic" platform is a powerful tool which provides comprehensive overview of individuals metabolic status which can not be obtained from transcriptomics and proteiomics. The first human metabolome draft was released in 2007. Metabolomics has been successfully applied in various research and clinical areas including early diagnosis of diabetes, cancer and cardiovascular diseases. Recently, attention has been focused on the novel concept of pharmacometabolomics, an emerging field that is derived from metabolomics. The current aspect of pharmacometabolomics knowledge shows promising potential and brighter prospect in the field of pharmacology especially in drug discovery, safety, toxicity, targeting, pharmacokinetics and pharmacodynamics. The pharmacometabolomics uses many modern tool of computational biology and combination of analytical technology including HPLC, LC-MS/MS, GC-MS/MS, NMR etc. Pharmacometabolomics is also focused on the use of individual metabolic signatures for the prediction and evaluation of drug efficacy and safety, eventually accelerating clinical pharmacology toward personalized drug therapy. It has proved its worthiness in identification of clinically useful biomarkers and biochemical processes to improve disease diagnosis and therapy. The current knowledge of pharmacometabolomics indicates its scope of application for diagnosis, prognosis, treatment and prevention of diseases in medical and veterinary fields.

Keywords: Pharmacometabolomics, metabolic profile, drug discovery, drug efficacy and safety, biomarker discovery, personalized medicine.

INTRODUCTION

Metabolomics is the scientific study of chemical processes involving the metabolites. These small metabolites are intermediates and/or end product of cellular processes and may reflect the metabolite phenotype, regulated by interaction between genotype and other environmental factors, including diet, lifecycle and gut microbiome. In this regard, the metabolomics approach provides a complete overview of an individual's metabolic state which cannot be obtained from transcriptomics and proteomics (Corona *et al.,* 2012). Metabolomics has significant potential in pharmaceutical and clinical research including identification of new targets, elucidation of the mechanism of action of new drugs, characterization of safety and efficacy profiles as well as the discovery of biomarkers for early disease diagnosis, prognosis, patient stratification and treatment response monitorization.

The growing and expanding applications of metabolomics and its brighter prospects in the area of pharmacology has resulted recently into emergence of newer branch of metabolomic science, known as 'Pharmacometabolomics'. The detailed study of pharmacometabolomics profile following the administration of a xenobiotic provides newer approach to identify mechanism of toxicity at early stage of drug discovery. The overall benefits of reducing cost and time in development and marketing of new drug is one of several hope for future. The use of metabolomics technologies to monitor mechanism of action and adverse drug reactions of drugs, and to stratify patient populations for clinical trials are major area of application.

Pharmacometabolomics refers to the direct measurement of metabolite in an individual's body fluids, in order to predict or evaluate metabolism of pharmaceutical compound and to better understand the pharmacokinetic profile of a drug (Kaddurah-Daouk and Wenshilboum, 2014). Pharmacometabolomics is an extension platform of 'omic research'. It takes care of variation in metabolites and metabolism to understand the different cellular processes at grass root level (Fig.1). It is different from genomics and transcriptomics as former involves study of structure and functions of genes which indicates possible error in drug metabolism. Human transcriptomics, made up of 85,000 transcripts, can provide information on important genes in metabolism being actively transcribed. Proteomics depicts kind of proteins which are active in the body to carry out these functions. Pharmacometabolomics provides the direct measurement of the product of all of these using genomics, proteomics and transcriptomics and provides integrated picture and final outcome at cellular and sub cellular level. The numbers of metabolons was initially projected to be approximately 2200 (Weiss and Kim, 2012), which may increase to a larger number if gut derived metabolites and xenobiotics are added to the list.

Alternatively, pharmacometabolomics can be applied to measure metabolite level following administration of a drug in order to monitor the effect of drug on certain

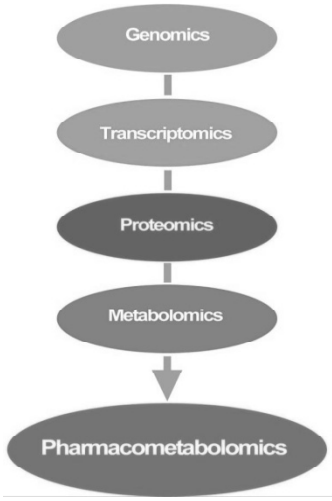

Fig: 1. Overwiew of the pharmacometabolomics process

metabolic pathways. This provides detailed mapping of drug effects on metabolism and the pathways that are implicated in mechanism of variation of drug response (Kaddurah-Daouk *et al.,* 2012; Yerges-Armstrong *et al.,* 2013). Thus, pharmacometabolomics has potential role in drug discovery for identification of novel drug target and pre-clinical evaluation of drug (Corona *et al.,* 2012). It has potential to provide an effective and inexpensive strategy to evaluate drug efficacy and safety, which make personalized medicine realistic both from scientific and financial perspectives (Xu *et al*., 2009; Malik *,* 2012).

The science of pharmacometabolomics encompasses the use of many modern analytical techniques including HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography - Mass Spectrometry), LC-MS (Liquid Chromatography Mass Spectrometry), NMR (Nuclear Megnatic Resonance), Crystallography, FT-MS (Fourier Transform ion cyclotron resonance Mass Spectrometry) and LIF (Laser-Induced Fluorescence) detection. This review addresses the current state of pharmacometabolomics, its application and future prospects in the fields of drug development and biomarker discovery, personalized medicine and drug response.

DRUG DISCOVERY AND DEVELOPMENT

Pharmacometabolomics has potential role in drug discovery and development for identification and validation of new drug, lead optimization, ADMET screening, pre-clinical studies, clinical studies and post approval studies. Drug discovery is directed toward the identification of biomarker of drug toxicity that can be used in preclinical and clinical studies of drugs (Corona *et al.,* 2012). Biomarkers predict toxicity in the preclinical development of new chemical entities (NCEs) early in the drug development process.

DRUG TARGET IDENTIFICATION AND VALIDATION

Pharmacometabolomics reveals new drug target and explore new treatment strategies. Pharmacometabolomics provides an excellent tool to study biochemical changes occurring in a particular disease, and then map these variations important for the development of a pathophysiological condition, in the corresponding metabolic network. Such study provides insight into detailed eluicidation of druggable disease targets and its interaction with drugs or vaccines. Metabolon study in experimentally induced ulcerative colitis revealed that stearoyl-CoA desaturase-1 (SCD-1) is a potential target in the treatment of the inflammatory disease (Chen *et al.,* 2008).

ADMET SCREENING

ADMET (absorption, distribution, metabolism, elimination and toxicity) could serve as screen test and optimization step in drug discovery and development. Pharmacometabolomics provides a faster, simpler, less invasive approach to characterize biopharmaceutical properties and toxicity as well as revealing their mechanism. In one study, NMR derived pharmacometabolomics analysis showed significant difference of cellular response at high and low doses of docetaxel in MCF7 breast cancer cells (Byet-robert *et al.,* 2010). The LC/MS based pharmacometabolomics approach applied to cisplastin's cytotoxicity in human lung cancer cell line has been also reported (Pyo *et al.,* 2008). These results demonstrated the efficacy of pharmacometabolomics in evaluation of cytotoxicity of various pharmaceutical ingredients and this has been widely accepted (Wei, 2011). Pharmacometabolomics phenotyping also reveals different response to xenobiotics in animals. Clayton *et al.* (2006) first demonstrated that a pre-dose metabolic profile of urine could predict the metabolism of paracetamol in rats. They evaluated NMR-based metabolomics profile of pre- and post-dose urine samples from rats given a single toxic dose of acetaminophen (APAP) to predict both individual susceptibility to APAP-induced toxicity, liver injury, and also relative excretion levels of APAP metabolites in the forms of glucuronide and sulfate (APAP-S) conjugates. NMR-based metabolomics discovered that human subjects with high predose levels of p-cresol (one of the metabolites related to an individual's gut microbiome) had lower concentrations of APAP-S, which is due to the competition of the binding site to the sulfotransferase enzyme. The individuals with lower predose urinary concentrations of p-cresol might be more susceptible to APAP-induced toxicity since less APAP will be conjugated with sulfate to produce APAP-S. The findings indicated that each individual, colonized by a unique assortment of trillions of microbes, could respond to a drug differently, either beneficially or adversely (Backshall *et al.,* 2011).

Pharmacometabolomics can be used to demonstrate the real possibility of using predose patterns

to develop models that could be used to predict toxicity and define the optimum therapeutic course for a patient. Application of NMR based Metabolomics profiling to patient suffering from locally advanced or metastatic colorectal cancer prior to treatment with capecitabin revealled correlation between distinct metabolomic patterns and occurance of adverse drug reaction.

PHARMACODYNAMICS

Metabolon has the ability to cluster drug candidates according to their common mechanism of action. This is useful in predicting the mechanism of unknown drug candidates. GC-MS based study on urine metabolomics in rats chronically poisoned with hydrogen sulphide demonstrated distinct metabolic profile in unexposed and chronically exposed rats.The level of alanine, d-ribose, tetradecanoic acid, L-aspartic acid, pentanedioic acid, cholesterol, acetate, and oleic acid in rat urine of the poisoned group were decreased as compared to unexposed or control. Mean level of glycine, d-mannose, arabinofuranose and propanoic acid was found to be increased in exposed rats (Deng *et al.,* 2015). This technique can be employed to decipher the mechanism of chronic H_2S poisoning, thus promoting the use of metabolomics in clinical toxicology.

PRE-CLINICAL STUDIES

Pharmacological effects occur in targeted tissues, but often they occur in non- targeted tissue and the undesired biochemical changes can lead to toxicity. Metabolon's technology differentiates drug leads based on their action and toxic effects on non-targeted tissues. Kamp *et al*. (2012) applied the *in vivo* metabolomics to pre-clinical studies on phenytoin induced systemic toxicity using Meta Map Tox (data base comprising of the metabolome of rat plasma). Metabolome analysis were performed on day 7, 14 and 28 days in rats given phenytoin @ 600 and 2400 ppm for 28 days. It is clear that metabolome analysis can provide more comprehensive picture of phenytoin toxicity as compared to conventional methods. It certainly adds value to pre clinical and toxicological studies.

CLINICAL STUDIES

Metabolon's approach to drug development can be potential tool to reduce time consumed in clinical trials. The duration of drug development can be shortened thereby making quicker availability of newer drug in the market. Mattes *et al.* (2013) studied the prediction of clinically relevant safety signals of nephrotoxicity of several drugs through plasma metabolite profiling. Metabolic profile of rats treated with phenytoin, cyclosporine-A, doxorubicin, captopril and lisinopril were established. Observed profiles were compared with available Meta Map Tox metabolomics database which predicted clinical toxicity of each compound.Such studies reveal that pharmaco-metabolomics offers powerful approach for augmenting the safety assessment and avoiding clinical adverse effects.

PERSONALISED MEDICINE

Pharmacometabolomics, together with pharmacogenomics is useful for achieving more effective and safe personalized medicine treatments. Personalized medicine provides the best medical treatment for each individual patient by determining which drug will have the best efficacy and have the least amount of toxicity and/or adverse effects (Everette *et al.,* 2013). Furthermore, understanding inter-individual variations of response to drug treatment, especially in patients with potential adverse reactions, might lead to biomarkers that can be used to predict the low incidence of idiosyncratic toxicity. Personalized medicine is usually based on the concept of pharmacogenomics. Despite enormous energy and monetary efforts, pharmacogenomics has limited success in clinical pharmacology to predict drug response with absolute certainty using single or multiple SNPs as biomarkers. The major reason for the limitation is that the response is dependent upon the phenotype of an individual, which is determined by both genotype and its complex interactions with other environmental factors. These environmental factors include diet, life style, gut microbiome, nutrition, medications (polypharmacy), age and exposures to toxins or dietary supplements, as well as the individual physical and pathological conditions (e.g., diabetes and obesity). Therefore, it is extremely critical to be able to access an individual's phenotype, which will provide useful information in determining the correct drug and dose treatment, and in predicting the potential response following a therapeutic intervention. Pharmacometabolomics is an emerging approach that combines metabolic profiling and chemometrics tools to link the inherent variation of a metabotype to the prediction of drug efficacy or toxicity in patients (Puchades-Carrasco and Pineda-Lucena, 2015). Personalized treatment is given based upon the disease phenotype whose beneficial outcome may be lower mortality rate and incidence of toxicity.

Pharmacometabolomics approaches will leads to the improve ability to predict an individual's response to a compound, the efficacy and metabolism of individual drug as well as adverse or off-target effects that may take place in the body. The metabolism of certain drugs varies from patient to patient as the copy number of genes which code for common drug metabolizing enzymes varies within the population, and leads to differences in the ability of an individual to metabolize different compound. Pharmacometabolomics analysis combined with pharmacogenetics, can function to identify the metabolic processes and particular genetic alteration that may compromise the anticipated efficacy of a drug in a particular patients. The result of such analysis can then allow for the modification of treatment regimens to optimize

treatment outcome. Pharmacometabolomics is an emerging approach that combines metabolic profiling and chemometrics tools to link the inherent variation of a metabotype to the prediction of drug efficacy or toxicity in patients. Phaple *et al.* (2010) evaluated the potential of a metabolic phenotype to predict individual variation in the pharmacokinetics of tacrolimus using four metabolites. The prediction of the plasma AUC (area under concentration –time curve) of tacrolimus in individualized pharmacokinetics appeared to be successful. The result indicated that integrative pharmacometabolomics approach combined with the metabolic profiling of pre-dose urine can serve as useful tool for personalized drug therapy.

Wikoff *et al.* (2013) studied the pharmaco-metabolomics to explain racial differences in response to atenolol treatment due to racial factors. Biochemical changes induced by the beta-adrenergic receptor blocker atenolol in Caucasians and African Americans were studied using a GC-TOF metabolomics platform. The metabolomic signature of atenolol exposure included saturated (palmitic), monounsaturated (oleic, palmitoleic) and polyunsaturated (arachidonic, linoleic) free fatty acids, which decreased in Caucasians after treatment, but were not different in African Americans (p,0.0005, q,0.03). Similarly, the ketone body 3- hydroxybutyrate was significantly decreased in Caucasians by 33% (p,0.0001, q,0.0001), but was unchanged in African Americans. The contribution of genetic variation in genes that encode lipases, responsible for camuing variation was also stidied. SNP rs9652472 in LIPC was found to be associated with the change in oleic acid in Caucasians (p,0.0005) but not African Americans, whereas the PLA2G4C SNP rs7250148 associated with oleic acid change in African Americans (p,0.0001) but not Caucasians. The findings from the study indicate that atenolol-induced changes in the metabolome are dependent on race and genotype. The pharmacometabolomics provides newer approach to phenotype patients with hypertension and gain mechanistic insights into racial variability in changes that occur with atenolol treatment, which may influence response to the drug.

Puskarich *et al.* (2015) studied pharmacometa-bolomics of L-carnitine treatment response phenotypes in patients with septic shock using untargeted 1H-nuclear magnetic resonance metabolomics. There were evidences which suggested distinct patterns of concentrations of 3-hydroxybutyrate, acetoacetate, and 3-hydroxyisovalerate at different time interval in L-carnitine treated survivors versus non-survivors, thus indicating impact of synthesis and degradation of ketone bodies on treatment response. L-Carnitine treated low-ketone patients had greater use of carnitine as evidenced by lower post-treatment L-carnitine levels. The L-carnitine responders also had faster resolution of vasopressor requirement and a trend toward a greater improvement in mortality at 1 year compared with patients with higher 3-hydroxybutyrate. The findings of the study suggests pharmacometabolomics as a viable strategy for informing L-carnitine responsiveness and a concrete example for the application of precision medicine to sepsis therapeutics for the benefits of patients.

Pharmacometabolomics has demonstrated extensive future possibility and prospect in identification of susceptible patients early in the treatment phase and before the onset of major adverse events. Winnike *et al. (2010)* evaluated pre- and post-dose urine metabolite profiles in healthy male and female volunteers that received 4 g of APAP over seven day period. There was a clear pattern of changes in urine metabolites after treatment indicating that APAP may induce a new metabolic phenotype that is measurable prior to classical toxicity end points.

BIOMARKER DISCOVERY

Metabolomics offers the capability to identify potential biomarker for diseases diagnostics and prognosis. The pharmacometabolomics can serve as diagnostic tool for cancer prevention, early detection, and monitoring the progression of diseases using novel pharmacological techniques like high resolution mass spectrometry and NMR. Shin *et al.* (2013) studied targeted metabolomics approach to evaluate endogenous biomarkers of cytochrome P450 (CYP) mediated drug metabolism. 4ß hydroxyl-cholesterol is an endogenous marker that is correlated with CYP3A activity. But it has limitation that it cannot effectively predict basal level and inhibition of enzyme. Now, newer approach using pharmaco-metabolomics like GC-MS based metabolomics analysis identified several endogenous markers having better predictive potential of CYP3A activity. Metabolomics is the discipline where endogenous and exogenous metabolites are assessed, identified and quantified in different biological samples. The technological developments in the field of NMR spectroscopy have enabled the identification and quantitative measurement of the many metabolites in a single sample of biofluids in a non-targeted and non-destructive manner. Combination of NMR spectra of biofluids and pattern recognition methods has driven forward the application of metabolomics in the field of biomarker discovery.

PHARMACOMETABOLOMICS IN CANCER STUDIES

Cancer is a disease that is known to alter cellular metabolism. Metabolomics can play a major role in early detection and diagnosis of cancer (Beger, 2013). Metabolomics has the ability to probe much further into the metabolism of biomolecules involoved in cancer and provide insight into how cancer cells use glycolysis to produce amino acids, nucleotides and lipids necessary for tumor proliferation and vascularization. Metabolomic processes are used in biomarker discovery in cancer

studies. This approach would also generate substantial data on druggability of newer molecular targets.

DRUG RESPONSE

Pharmacometabolomics helps to predict individual drug response and variation. The mapping of drug metabolite signatures in good and poor responders could also identify pathways of importance causing variation in response to therapy. The optimum concentration of drug is determined by pharmacokinetics and pharmacodynamics processes. Pharmacometabolomics helps us to understand the detailed aspect of pharmacokinetic and pharmacodynamic processes.

Trupp *et al*. (2012) found that metabolite profiling can successfully separate patients into two response groups and could predict LDL-cholesterol response related to simvastatin treatments. Simvastatin reduces cholesterol synthesis and lowers LDL level by inhibiting enzyme 3-hydroxyl-3-methy-glutaryl-CO-A. It reduces synthesis of many inflammatory intermediates. It has been found that approximately 50-80% treated patient showed residual risk of CVD and shows multiple toxicity effects. Pharmacometabolomics study proved useful to make clear many of this mechanism. In one clinical trial of six week duration, 944 patients having plasma cholesterol level ranging between 160 to 400 mg/kg from varied background were selected and treated with dose of 40 mg/kg daily. Pharmacometabolomics profiles of all the patients were established and analysed. More than 300 lipid species were included in metabolomics study. There were distinct patterns of metabolom in good and bad responders. Lower level of xanthine, 2-hydroxyl valeric acid and 2-hydroxyl pentanoic acid were observed in good responders as compared to values observed in poor responders. The mapping of effects of these changes suggests variable response of statin therapy. Statin treatment increased the level of arachidonic acid because of up regulation of fatty acid desaturase. Arachidonic acid is a precursor of many pro and anti-inflammatory mediators. So, it was concluded that it may be possibly involved in both clinical efficacy and adverse effects (Trupp *et al*., 2012).

APPLICATIONS IN VETERINARY FIELDS

The science of pharmacometabolomics is recently steamed up metabolomic platforms. The research in veterinary field is gaining momentum. It has great scope of application in development of veterinary drugs, specific markers for diagnosis of animal diseases, identification of biomarkers for residue analysis of antibiotics and growth promoters. Growth-promoting agents are continually misused for increasing animal growth and fraudulent gain in the meat industry. The current technology to detect and identify these depend on qualitative and quantitative analysis of such compounds or their intermediates. But metabolomics or pharmacometabolomics provides a more confident tool of biomarkers identification for the issue based on screening of biological responses of an animal to such growth-promoting agents. An untargeted proteomics approach using comparative two-dimensional gel electrophoresis (2-DE) was carried out to identify putative proteins altered in plasma after treatment with oestradiol, dexamethasone or prednisolone (Kinkead *et al.,* 2015).

Fatty liver is a common metabolic disorder of dairy cows during the transition period. Current practices employed for diagnosis of fatty liver includes liver biopsy, biochemical or histological examination of liver specimens, and ultrasonographic imaging of the liver. The Metabolomics has potentiality to provides substitutes for existing techniques which are convenient and non-invasive. The investigation of plasma metabolic profiles of dairy cows with fatty liver and normal (control) to identify new biomarkers using nuclear magnetic resonance revealed the potentiality of metabomic science.

Such ^{1}H-nuclear magnetic resonance-based metabolomics coupled with pattern recognition analytical methods also has been successfully attempted earlier to specifically distinguish cows with clinical and subclinical ketosis, a metabolic disorder, from healthy controls and, also has the potential to be developed into a clinically useful diagnostic tool that could contribute to a further understanding of the disease mechanisms (Sun *et al.*, 2014).

LIMITATIONS OF PHARMACOMETABOLOMICS

Meaningful metabolic signatures can be elucidated to create baseline value. The generation of base line values needs integrated approach and huge financial resources. Issues surrounding the measurement of metabolites in an individual can arise from the methodology of metabolite detection. There are arguments both for and against NMR and mass spectrometry because of cost and complexities of technology. Metabolite analysis includes proper handling and processing of sample, as well as proper maintenance and calibration of the analytical and computational equipment. These tasks require highly skilled and experienced technicians and expertise. The considerable instrument repair costs involved in continuous sample processing is the major hindrance for its wide applicability. The cost of processing and analytical platforms alone is too high, making it difficult for many facilities to afford pharmacometabolomics- based treatment analyses.

CONCLUSIONS

Pharmacometabolomics is new omic platforms having future potential in the field of pharmaceutical research and development. It helps to understand detailed pharmacokinetics and pharmacodynamic processes which determines the efficacy and safety of new drug. It can be applied in the field of drug discovery *i.e.* identification of targets, optimization of lead, pre-clinical and clinical

screening. It is capable of predicting toxicity which could not be detected during pre-clinical and clinical trials. The concept of personalized medicine has been made realistic with knowledge of pharmacometabolomics, as it could well understand mechanism responsible for variation in drug response at individual as well as population level. It is also useful in the field of cancer diagnosis, prognosis and therapeutics. Identification of biomarkers for diagnosis, prognosis, monitoring of diseases progression and regression, drug response and efficacy has been explored with the help of pharmacometabolomics. Thus, the budding science of pharmacometabolomics has potential of bright prospects in pharmacological science.

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Research Article

EFFECT OF MELOXICAM CO-ADMINISTRATION AND FEBRILE STATE ON PHARMACOKINETIC OF CEFQUINOME IN GOATS

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ABSTRACT

The pharmacokinetic of cefquinome (20 mg kg^{-1}) was studied following intramuscular administration of cefquinome alone, co-administered with meloxicam (0.2 mg kg^{-1}) and under *Escherichia coli* lipopolysaccharide induced febrile state in goats. The concentration of cefquinome in plasma was detected by HPLC. Following single dose intramuscular administration of cefquinome alone, peak plasma concentration (7.16±0.13 µg mL^{-1}) was obtained at 0.75 h. The absorption half-life ($t_{1/2K\alpha}$), volume of distribution (Vd_{area}), total body clearance (Cl_B), elimination half-life ($t_{1/2\beta}$), area under plasma drug concentration-time curve ($AUC_{(0-\infty)}$) and area under first moment curve (AUMC) of cefquinome were 0.15±0.01 h, 2.58±0.22 L kg^{-1}, 1.02±0.09 L h^{-1} kg^{-1}, 1.75±0.08 h, 17.16±0.42 µg.hmL^{-1} and 41.89±1.37 g.h^2/mL, respectively. No significant changes were reported in pharmacokinetic parameters following co-administration of cefquinome with meloxicam. Following intramuscular administration of cefquinome in febrile goats, significant increase ($p<0.05$) in plasma drug concentration of 8.55±0.16, 1.98±0.06 and 0.87±0.05 µg/mL observed at 0.75, 4 and 6 h., respectively, as compared to healthy goats. The pharmacokinetics parameters like $Vd_{(area)}$ (1.69±0.06 L kg^{-1}), $Cl_{(B)}$(0.78±0.02 L h^{-1} kg^{-1}) and $t_{1/2\beta}$ (1.47 ± 0.06 h) were significantly lower ($p<0.05$) than non-febrile goats. A significant ($p<0.01$) increase in C_{max}(9.38±0.08 µg ml^{-1}), AUC (24.58±0.29 µg.hmL^{-1}) and AUMC (57.14±1.63µg.h^2 mL^{-1}) were observed in febrile goats as compared to healthy goats. Pharmacokinetic data of cefquinome generated from the present study and MIC value of cefquinome sensitive bacteria suggested that the drug can be administered intramuscularly (20 mg kg^{-1}) with meloxicam and in febrile condition at 8 h. interval to combat susceptible bacterial infections in goats.

Keywords: cefquinome, goats, meloxicam, pharmacokinetics.

INTRODUCTION

Antimicrobials and NSAIDs are used most frequently in multiple drug prescription. Co-administration of several drugs often results in either diminished therapeutic efficacy or increased toxicity of one or more of the administered drugs.

Cefquinome is a new fourth generation aminothiazolyl cephalosporin which has been developed solely for veterinary use. Cefquinome has the zwitter-ionic property which facilitates rapid penetration across biological membranes including the porins of the bacterial cell wall, enhance bioavailability and improve the spectrum of antimicrobial activity compared with the second and third-generation cephalosporins (Thomas *et al*., 2006). It has a broad spectrum of activity and susceptible to various clinically important bacteria such as *Streptococcus spp., Staphylococcus spp., Pseudomonas spp., Moraxella spp., Haemophyllus, Corynebacterium, Enterococci, Escherichia coli* and gram positive anaerobes. It is used for treatment of respiratory tract diseases, acute mastitis and foot rot in cattle, calf septicemia, and respiratory diseases in pigs, metritis-mastitis-agalactia syndrome in sows, foal septicemia and respiratory tract diseases in horses (CVMP, 2005).Meloxicam, a novel NSAID of the oxicam class, is one of the most potent inhibitors of inducible cyclooxygenase-2 (COX-2) and have anti-inflammatory, analgesic and antipyretic activities. Pharmacokinetics of cefquinome has been investigated in goats and other species of animals (Dumka *et al.,* 2013; Song *et al.,* 2012; Sagar *et al*., 2013 Shalaby *et al.,* 2015), However, there is no information available on the influence of co-administration of meloxicam and febrile state on the pharmacokinetics of cefquinome in goats, therefore, the study was undertaken to determine effect of meloxicam and febrile state on pharmacokinetics of cefquinome in goat.

MATERIALS AND METHODS

Experimental animals

The experiment was conducted on six Surti goats of 1-2 years of age and weighing between 15.0 - 20.0 kilograms. The animals were housed in separate pens andprovided standard ration with *ad libitum* water. Goats were kept under constant observation for two weeks before the commencement of the experiment and subjected to clinical examination to exclude the possibility of any diseases. This study was undertaken after approval of IAEC.

Drug and chemical

Pure cefquinome sulphate powder (99.99%) was obtained from Sigma-Aldrich Pvt. Ltd., Mumbai, India. Cefquinome sulphate injection (25 $mgml^{-1}$; Cobactan 2.5%®Intervet India Pvt. Ltd., Pune, India) and Meloxicam injection (5$mgml^{-1}$; Melonex®; Intas Pharmaceuticals Ltd.,

Ahmedabad, India) were procured from local market. Acetonitrile, Trifluoroacetic acid and Methanol (HPLC grade)were purchased from Merck India Ltd. Lipopolysaccharide (LPS) of *Escherichia coli* was purchased from Sigma-Aldrich Pvt. Ltd., Mumbai, India.

Drug administration and sample collection

The study was planned in cross over design to receive either an intramuscular injection of cefquinome alone at the dose rate of 20 mg kg^{-1} or intramuscular injection of cefquinome(20 mg kg^{-1}) along with Meloxicam (0.2 mg kg^{-1}) in each six healthy goats with a 15 days washout period between administration. The intramuscular injection of cefquinome was administered in the left deep gluteal muscle, while meloxicam was administrated deep intramuscular at the dose rate of 0.2 mg kg^{-1} in contralateral gluteal muscle. Blood samples (2 mL) were collected, before administration and at 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, 8, 12, 18, 24 and 36 h after concurrent intramuscular administration of cefquinome and meloxicam. Febrile state in goat was induced by injecting Lipopolysaccharide (LPS) of *Escherichia coli* at the dose rate of 0.2 µg kg^{-1} body weight intravenously. LPS was repeated at dose rate of 0.1 and 0.05 µg kg^{-1} at 12 and 24 h, respectively to maintain the febrile state up to 36 h. Goats were monitored for any adverse reactions during the entire study period. The blood samples were centrifuged (4116 g for 10 minutes). The plasma samples were transferred to cryo-vials (2 ml) and then stored at –4°C. Samples were analyzed within 24h to quantify cefquinome concentration using HPLC(Adept Cecil Instrument, England).

Analysis of cefquinome and pharmacokinetics

Cefquinome was assayed in plasma with modification of procedure described by Uney *et al.* (2011). Separation was performed by using reverse phase C18 column (4.6 × 250 mm) at room temperature. The mobile phase was a mixture of 0.1% trifluoroacetic acid and acetronitrile in the ratio of 85:15 with a pH of 3.94. Mobile phase was filtered by 0.2 µ filtersand pumped into column at a flow rate of 1.5 ml min^{-1} at ambient temperature. The effluent was monitored at 268 nm wavelength. Plasma proteins were precipitated by addition of a solution containing methanol and water (4:1). Two hundred microliter (200µl) of plasma sample and 400 microliter of methanol: water (4:1) was mixed in a clean centrifuge tube by shaking on a vortex mixer for 1 minute and followed by centrifugation for 10 minutes at 4116 g. The clean supernatant was collected and an appropriate aliquot was injected into the HPLC system through auto injector.

Calibration curve was prepared daily for drug concentration ranging from 0.16 to 166.6 µg ml^{-1} and the assay was found sensitive, reproducible and linear with R^2 = 0.998. The limits of detection and limits of quantification were determined to be 0.04 and 0.08 µg ml^{-1}, respectively. Precision and accuracy were determined using quality control (QC) samples at concentrations 0.2, 2, 5,10 and 20 µg ml^{-1}(3 replicates of each per day). The intraday and inter-day coefficients of variation for 5 QC samples were satisfactory with the relative deviations (RSD) of less than 14.65 %. Pharmacokinetic parameters were calculated from plasma concentration of cefquinome using software PK solution (version 2.0).

Statistical analysis

Cefquinome plasma concentration and pharmacokinetic parameters of different treatment groups were compared by “students ’t” test using MS Excel (version 2007).

RESULTS AND DISCUSSION

Plasma cefquinome concentrations at different time intervals following intramuscular injection alone, cefquinome co-administered intramuscular with meloxicam and cefquinome under febrile state in goats is presented as semi logarithmic plot in Figure-1.

The plasma concentration of cefquinome alone at 5 min. after intramuscular administration was 1.93±0.08µg mL^{-1} which gradually increased and reached to the peak concentration (7.16±0.13µg mL^{-1}) at 45 min. On concurrent intramuscular administration of meloxicam and cefquinome, the initial plasma concentration of cefquinome at 5 min was 1.95±0.28µg mL^{-1} which increased to attain the peak plasma concentration (7.82±0.44 µg mL^{-1}) at 45 min. In febrile condition, plasma cefquinome concentration following intramuscular injection was 4.17±0.11µg mL^{-1} at 5 min. which attained peak concentration at 30 min. (9.38±0.08µg mL^{-1}). Various kinetic determinants that describe the absorption and elimination pattern of cefquinome after intramuscular administration either used alone or in combination with meloxicam and under febrile state were calculated and are presented in Table 1.

Following intramuscular administration cefquinome (20 mg kg^{-1}) in goat either alone and co-administered with meloxicam (0.2 mg kg^{-1}) no adverse effects or toxic manifestations were observed. In endotoxin induced febrile condition symptoms viz., increased respiration and pulse rate, decrease in feed intake, dryness of mouth and muzzle and incoordination in movements were observed. Peak plasma cefquinome concentration (C_{max}) observed in meloxicam co-administrated goats was not altered significantly as compared to goats given cefquinome alone. Our findings were consistent with the findings of Patel *et al*. (2012) who reported that peak serum cefepime concentration (C_{max}) observed in ketoprofen co-administrated goats was similar to goats given cefepime alone. In contrast, variations in pharmacokinetics of different cephalosporins have been observed following concurrent administration with NSAIDs in cross-bred calves following concomitant intramuscular administration of paracetamol

with ceftizoxime (Singh *et al*., 2008; 2013). In febrile goats, significantly higher cefquinome peak concentration was observed as compared to non-febrile goats. Decreased peak concentration of cefazolin following intramuscular administration was reported in febrile goats in comparison to cefazolin in non-febrile goats (Roy *et al*., 1994).

Pharmacokinetic parameters were not altered significantly following intramuscular administration of cefquinome with meloxicam in goats in comparison to goats given cefquinome alone. Pharmacokinetic parameters of cefepime also remained unchanged following concurrent administration of ketoprofen in goats (Patel *et al*., 2012) which supports results of our study. However, significant decrease in body clearance, volume of distribution, elimination half-life and absorption half-life were found after concurrent intramuscular administration of ceftriaxone with acetaminophen in febrile goats (Jimoh *et al*., 2011). Following intramuscular administration of cefquinome in febrile goats, significant increase in AUC and AUMC has been observed as compared to healthy goats. Sagar *et al*. (2013) also found significant increase in AUC and AUMC of cefquinome in febrile goats after intravenous administration as compared to healthy goats which supports results of our study. The values of $Vd_{(area)}$, $Cl_{(B)}$ and $t_{1/2\beta}$ were significantly lower ($p<0.05$) in febrile goats as compared to that of non-febrile goats. The values of $t_{1/2ka}$, MRT and MAT were not significantly altered following intramuscular administration of cefquinome in febrile compared to healthy goats. The findings indicate that administration of lipopolysaccharide modulates the elimination of the drug which could be due to organ (renal and hepatic) modifications caused by the toxin. Endotoxin induces toxic and adverse effects on the kidneys, including direct vascular damage to the endothelium and platelet aggregation in renal glomerular capillaries. It also produces some functional changes including decrease in the renal blood flow and glomerular filtration rate and changes in the intra-renal hemodynamics (Hasegawa *et al*., 1999). Following intramuscular administration of cefalosporins in febrile goats, along with elimination half-life, volume of distribution was significant increased while significant decrease in the value of area under curve was reported (Roy *et al*., 1994; Singh et al., 2013). In other study significant decrease in body clearance, volume of distribution, elimination half-life after single intramuscular administration of ceftriaxone were found in febrile goat (Jimoh *et al*., 2011) which is in line with our study. Variation in pharmacokinetic parameters of cefepime and other cephalosporins along with NSAIDs and in febrile condition may be due to differences in chemistry of drugs and species.

Cefquinome is a beta lactam antimicrobial and act as a time dependent bactericidal drug (Thomas *et al.,* 2006). For β-lactam antibiotics, many authors have

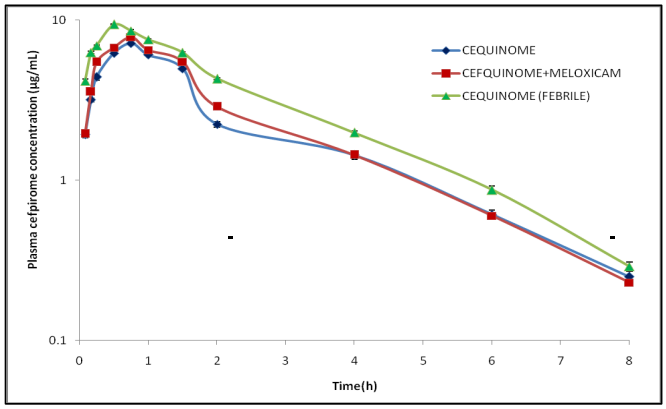

Fig 1:
Semilogarithmic plot of plasma concentrations of cefquinome after intramuscular (20 mg/kg) in healthy, meloxicam-treated (0.2mg/kg) and febrile goats (LPS-induced). Each point represents mean ± S.E of six animals.

Table 1:
Comparison of pharmacokinetic parameters (Mean ± S.E.) of cefquinome after intramuscular administration (20 mg/kg) in healthy, Meloxicam-treated (0.2 mg/kg) and febrile goats (n = 6).

Pharmacokinetic parameter	Unit	Goats		
		Healthy (Non-febrile)	Meloxicam-treated	Febrile
K_a	h^{-1}	4.57±0.28	4.37±0.26	3.50±0.80
β	h^{-1}	0.40±0.02	0.44±0.53	0.47±0.02
$t_{½Ka}$	h	0.15±0.01	0.16±0.01	0.27±0.06
$t_{½\beta}$	h	1.75±0.08	1.60±0.05	1.47±0.06*
C_{max}	µg mL^{-1}	7.16±0.13	7.76±0.39	9.38±0.08**
T_{max}	h	0.75±0.00	0.75±0.00	0.50±0.00
$AUC_{(0-\infty)}$	µg.h mL^{-1}	17.16±0.42	18.49±0.74	24.58±0.29**
AUMC	µg.h^2 mL^{-1}	41.89±1.37	41.44±1.27	57.14±1.63**
$Vd_{(area)}$	L kg^{-1}	2.58±0.22	2.28±0.18	1.69±0.06*
$Cl_{(B)}$	mL min^{-1} kg^{-1}	1.02±0.09	0.97±0.09	0.78±0.02*
MRT	h	2.44±0.06	2.25±0.05	2.32±0.04
MAT	h	0.61±0.07	0.41±0.06	0.49±0.02

*Significant at $p<0.05$, **highly significant at $p<0.01$ when compared with respective values of healthy/Non-febrile goats.

advocated the time for which plasma drug concentration exceeds the MIC (% T>MIC) of pathogens as a primary determinant of antibacterial efficacy (Turnidge, 1998).The most important pharmacodynamic/pharmacokinetic parameter for this type of drug is the length of the time during which drug remains above MIC_{90} value (Turnidge,1998; Mckellar *et al*.,2004). It is generally recommended that % T>MIC should be at least 50 to 70% of the dosage interval to ensure an optimal bactericidal effect (Toutain and Lees, 2004).

The minimum inhibitory concentration of cefquinomefor a majority of cefquinome sensitive bacteria is in the range of 0.016 to 0.781 µg ml^{-1} (Thomas *et al.,* 2006; Zonca *et al*., 2011). The drug levels above the Minimum Inhibitory Concentration (MIC) were detected in plasma up to 6 h following single dose intramuscular administration of cefquinome alone, co-administrated with meloxicam and in febrile state.

It can be concluded that cefquinome can be successfully co-administrated with meloxicam (0.2mg kg^{-1}) for combating inflammatory conditions @ 20 mg kg^{-1}, IM, at 8 h interval is sufficient in goat to maintain 70%T>MIC for bacteria with MIC values <0.8 µgml^{-1}.

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Research Article

INFLUENCE OF ACIDOSIS ON HISTAMINERGIC RECEPTORS IN SUPERIOR MESENTERIC ARTERY OF GOAT (*CAPRA HIRCUS*)

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ABSTRACT

The present study aimed to evaluate the effect of *in vitro* acidosis (extracellular) on histamine-induced vasocontractile response in goat superior mesenteric artery (GSMA). Histamine-induced concentration related contractile (CRC) response curve elicited at pH_o 7.4 (E_{max} 2.18±0.24g, pD_2 5.58±0.20) was attenuated with rightward shift of CRC response curve at acidic pH_o (6.8, 6.0, 5.5, 5.0, 4.5). Single dose 10µM Histamine-induced contractile response was also proportionately decreased (1: 0.67: 0.50: 0.45: 0.19: 0.06) with decrease in pH_o 7.4'!6.8'!6.0'!5.5'!5.0'!4.5. Endothelium denudation increased the contractile response to Histamine (10µM) by 35% at pH_o 7.4, by 60% at pH_o 6.0 and 77% at pH_o 5.0 as compared to their respective ED+ GSMA rings. Hence, acidosis attenuated the histamine mediated contractile response in GSMA that is primarily due to reduced function of histaminergic receptors and partially due to increased release of endothelium dependent relaxing factors.

Keywords: Histamine, pH_o, endothelium, superior mesenteric artery, *Capra hircus.*

INTRODUCTION

Histamine is an important modulator of sympathetic neurotransmission participating in many cell physiological processes such as allergic reaction, inflammation, gastric acid secretion, central and peripheral neurotransmission (da Silva Júnior *et al.,* 2014). It also maintains a local regulatory role in vascular smooth muscle tone, cardiovascular collapse and vascular shock. Histamine can elicit vasoconstriction or vasodilation depending on the cell types e.g., smooth muscle cells, endothelial cells etc on which it is acting through H_1, H_2, H_3, or H_4 receptors. Histamine elicited a contractile response in mesenteric artery, aorta and ciliary arteries of rat and bovines (Broadley *et al.,* 2013). Though previous studies have shown a variation in responsiveness and sensitivity of the different regions of the gastrointestinal tract to histamine, till date, no report is available on the characterization of histaminergic receptors in goat superior mesenteric artery (GSMA).

Acidosis can affect the agonist-induced vasoconstriction by increasing or decreasing or no change in the maximal response. Thus, the effect of pH upon vasocontractile mechanism is often disparate and may vary depending upon species, strain, vascular location and calibre and experimental model (Celotto *et al.,* 2011). Hence, the present study examined the effect of acidosis on histamine induced contractile response in GSMA rings.

MATERIALS AND METHODS

The GSMA of a freshly slaughtered goat was collected from the local slaughterhouse in cold (4º–6ºC) oxygenated modified krebs-henseleit solution (MKHS). The arterial rings were cut into 1.5-2 mm long circular rings and further employed for isometric contraction studies. Endothelium was removed by cotton swab method (Rosolowsky *et al.,* 1991). After equilibrating the arterial ring in MKHS adjusted to pH_o 7.4, 6.8 or 6.0 or 5.5 or 5.0 or 4.5 by using 1N HCl (Celotto *et al.,* 2011) for 45 min, histamine (1ηM 100mM) was added to bath in a cumulative manner at an increment of 1 log unit at 4 min interval to obtain concentration-related contractile response. To determine the influence of endothelium, submaximal dose of histamine (10µM) was added to the 20ml bath in both endothelium intact (ED+) and denuded (ED-) GSMA rings. Net tension (gm) due to each concentration was recorded and compared at different pH_o. E_{max}/ E_{Bmax} or pD_2 were analysed using Graph-Pad prism 5 software (San Diego, CA, U.S.A) and compared using 2 way ANOVA followed by Bonferroni's least significant difference post hoc test (Graph-Pad prism 5 software). A 'p' value < 0.05 was considered statistically significant.

RESULTS

The histamine induced concentration related contractile response curve elicited at pH_o 7.4 (E_{max} 1.57±0.05g, pD_2 4.19±0.14, n=12) was shifted to right with significant ($P<0.05$) decrease in E_{max} and increase in pD_2 at pH_o 6.8 (E_{max} 0.92±0.02g; pD_2 4.84±0.13, n=6); shifted to right with significant ($P<0.05$) decrease in E_{max} and pD_2 at pH_o 6.0 (E_{max} 0.69±0.07g, pD_2 4.01±0.33, n=14), pH_o 5.5 (E_{max} 0.68±0.09g, pD_2 4.38±0.22, n=8), pH_o 5.0 (E_{max} 0.41±0.09g, pD_2 4.88±0.27, n=20) and pH_o 4.5 (E_{max} 0.12±0.01g, pD_2 4.55±0.13, n=8) respectively (Table 1 and Fig. 1). Similarly, submaximal dose of histamine (10µM) induced contractile response at pH_o 7.4 (1.54±0.16g) was significantly ($P<0.05$) decreased at pH_o 6.8 (1.03±0.11g),

Table 1:
Histamine (1sM 100μM)-induced contractile response at pH_o 7.4, 6.8, 6.0, 5.5, 5.0 and 4.5 in goat mesenteric artery. Data were expressed as mean gram tension±SE.

		pH 7.4	pH 6.8	pH 6.0	pH 5.5	pH 5.0	pH 4.5
Histamine	E_{max}	1.57±0.05 (n=12)	0.92±0.02[a] (n=6)	0.69±0.07[a] (n=14)	0.68±0.0[a] (n=8)	0.41±0.09[a] (n=20)	0.12±0.0[a] (n=8)
	pD_2	4.19±0.14	4.84±0.13	4.01±0.33	4.38±0.22	4.88±0.27	4.55±0.13

n=Number of experiments, [a]P<0.05 versus pH 7.4 (control)

Table 2:
Histamine (10μM)-induced contractile response at pH_o 7.4, 6.8, 6.0, 5.5, 5.0 and 4.5 in GSMA rings. Data were expressed as mean gram tension±SE.

	Mean gm tension					
	pH 7.4	pH 6.8	pH 6.0	pH 5.5	pH 5.0	pH 4.5
Histamine (n=6)	1.54±0.16	1.03±0.11[a]	0.78±0.05[a]	0.70±0.19[a]	0.30±0.01[a]	0.09±0.01[a]

n=Number of experiments, [a]P<0.05 versus pH 7.4 (control)

Table 3:
Histamine (10μM)-induced contractile response at pH_o 7.4, 6.0 and 5.0 in goat mesenteric artery. Data were expressed as mean gram tension±SE.

	E_{max}	
TREATMENT	ED+	ED-
pH 7.4 (n=6)	1.41±0.06	1.91±0.04[a]
pH 6.0 (n=6)	0.88±0.21	1.47±0.01[a]
pH 5.0 (n=6)	0.27±0.01	0.48±0.06[a]

n=Number of experiments, [a]P<0.05 versus ED+ (control)

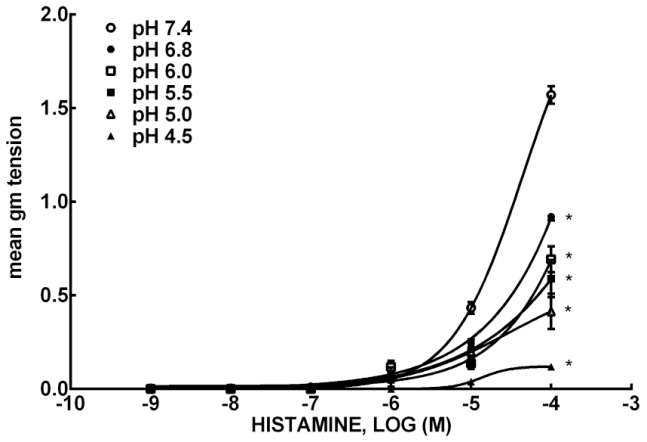

Fig. 1:
Histamine (1žM 100μM)-induced concentration related contractile (CRC) response curve in GSMA rings maintained at pH_o 7.4, 6.8, 6.0, 5.5, 5.0 and 4.5. *P<0.05 versus pH 7.4 (control).

pH_o 6.0 (0.78±0.05g), pH_o 5.5 (0.70±0.19), pH_o 5.0 (0.30±0.01 g) and pH_o 4.5 (0.09±0.01g) (Table 2 and Fig. 2). Endothelium denudation significantly (p<0.05) increased the histamine (10μM) induced contractile response at pH_o 7.4 (1.91±0.04g), pH_o 6.0 (1.47±0.01g) and pH_o 5.0 (0.48±0.06g) as compared to ED+ GSMA rings (pH_o 7.4: 1.41±0.06g; pH_o 6.0: 0.88±0.21g; pH_o 5.0: 0.27±0.01g) (Table 3 and Fig. 3).

DISCUSSION

In present study, we observed that, histamine (1μM-100μM) elicited a concentration dependent contractile response with threshold concentration 10μM, maximal response 1.57g and pD_2 3.97 in GSMA rings at pH 7.4. This contractile response to histamine appears to involve the activation of the H_1 receptor (Martý´nez *et al.,*

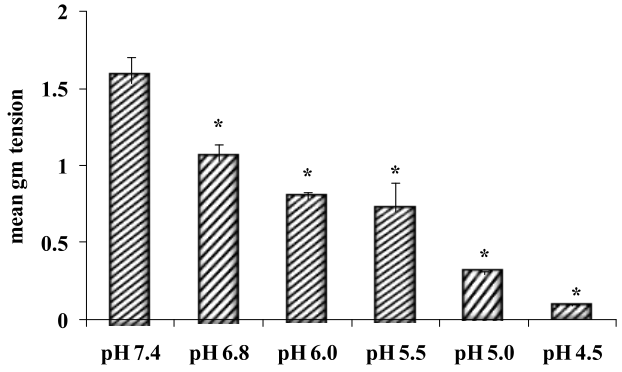

Fig. 2:
Histamine (10μM)-induced contractile response in GSMA rings maintained at pH_o 7.4, 6.8, 6.0, 5.5, 5.0 and 4.5. *P<0.05 versus pH 7.4 (control).

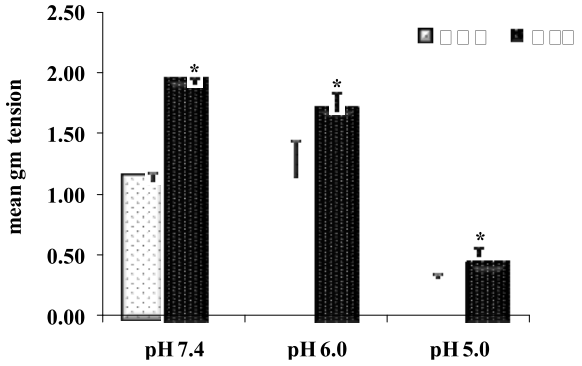

Fig.3:
Histamine (10μM)-induced contractile response in GSMA rings in endothelium intact and denuded GSMA rings maintained at pH_o 7.4, 6.0 and 5.0. *P<0.05 versus ED+ (control).

2002), mediated by increase in cytosolic Ca^{2+} influx through voltage dependent calcium channel (VDCC) or via IP_3 mediated $[Ca]_i^{2+}$ pooling or activation of nonselective cation channels (NSCC) (Kuriyama *et al*., 1995). Endothelium denudation increased the contractile response by 35% as compared to endothelium intact rings at pH 7.4, thus suggesting that histamine induced contraction is partially endothelium-dependent in GSMA rings as observed in bovine intrapulmonary vein (Gruetter *et al.,* 1994).

Reduction of pH_o (7.4'!6.8'!6.0'!5.5'!5.0'!4.5) attenuated the Histamine induced mean maximal response (E_{max}) in CRC curve to 59%, 44%, 43%, 26% and 8% of the control (pH_o 7.4) at pH_o 6.8, 6.0, 5.5, 5.0 and 4.5. Histamine (10µM) induced contractile response was significantly and proportionately decreased (1:0.67:0.50:0.45:0.19:0.06) with decrease in pH_o 7.4'!6.8'!6.0'!5.5'!5.0'!4.5. Thus, histamine-induced contraction was attenuated proportionately in a dose dependent manner with decrease in pH 7.4'!6.8'!6.0'!5.5'!5.0'!4.5 which may be due to reduced sensitivity of L Type Ca^{2+} channels as reported in rabbit basilar arteries (Kim *et al*., 2004) and GSMA (Mohanty and Parija, 2015) or due to altered sensitivity of histaminergic receptors (Flavahan and McGrath, 1981). Endothelium denudation significantly increased the contractile response to Histamine (10µM) by 35% at pH_o 7.4, by 67% at pH_o 6.0 and 77% at pH_o 5.0 as compared to their respective endothelium intact GSMA rings suggesting that with decrease in pH there is increase in endothelial function and increase in nitric oxide production (Pedoto *et al*., 2001) which partially contributed to attenuation of H_1 receptor mediated vasotonic response.

In conclusion, acidosis attenuated the histamine mediated contractile response in GSMA that is primarily due to reduced function of histaminergic receptors and partially due to increased release of endothelium dependent vasorelaxation factors (both EDRF and EDHF), thus, ultimately producing significant reduction in vascular resistance under acidosis.

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Research Article

MODULATION OF INSULIN RELAXATION OF GOAT INTERNAL ILIAC ARTERY IN PRESENCE OF ODQ BY ACETYLCHOLINE

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ABSTRACT

This study was undertaken to investigate the modulatory role of insulin in combination with 1H-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one (ODQ) on ACh induced vasorelaxation in 5-hydroxytryptamine (5-HT) and phenylephrine (PE) precontracted goat internal iliac artery (GIIA) rings. Ach (1sM-100μM) induced vasorelaxation was elicited in presence of insulin or ODQ+Insulin or ODQ+Insulin+4AP in PE and 5HT precontracted GSMA rings by measuring isometric tensions in GIIA. ACh induced concentration related contractile response in PE pre-contracted GIIA rings was shifted to right with significant ($p<0.001$) decrease in mean pD_2 and unaltered E_{Bmax} in presence of insulin and significant ($p<0.001$) decrease in mean pD_2 and E_{Bmax} in presence of INS+ODQ, INS+ODQ+4-AP as compared to control. In presence of insulin, ACh induced concentration related contractile response in 5HT pre-contracted GIIA rings with significant ($p<0.05$) increase in pD_2 and E_{max} value whereas in presence of INS+ODQ and INS+ODQ+4-AP the curve shifted to right with significant ($p<0.05$) increase in pD_2 and decrease in E_{Bmax} of INS+ODQ , INS+ODQ+4-AP as compared to control. Thus, insulin slightly increased the affinity and increased the vasorelaxation effect of ACh in both 5-HT and PE pre-contracted GIIA rings. It was also concluded that ODQ significantly blocked ACh response in insulin pretreated GIIA indicating dependency of insulin mediated relaxation e on sGC activated cGMP.

Keywords: 4-AP, goat internal iliac artery, insulin, nitric oxide, ODQ.

INTRODUCTION

Impairment of insulin-induced microvascular effects may contribute to obesity-associated insulin resistance (Clerk *et al*., 2006). In order to reduce the resistance of insulin, the possible pathway which is responsible for insulin relaxation should be known. Endothelium-derived nitric oxide (NO) is a gas that is synthesized from the precursor L-arginine in a reaction catalyzed by NO synthase and continuously released from the endothelium (Moncada *et al*., 1988). Ignarro (1990) demonstrated that NO released from the endothelium diffuses via the subendothelial space to the vascular smooth muscle where it binds to the heme group of guanylate cyclase and stimulates the generation of cyclic GMP (cGMP), which, in turn, leads to a decrease in intracellular Ca^{2+} levels resulting in smooth muscle relaxation and vasodilation. It has also been documented that insulin exerts its vascular effects mainly through the stimulation of NO synthesis. (Zeng and Quon, 1996).

1H-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one (ODQ) is highly selective, irreversible, heme-site inhibitor of soluble guanylyl cyclase (Schrammel *et al*., 1996; Teixeira *et al*., 2006). The binding of ODQ is competitive with NO. The inhibition of soluble guanylyl cyclase is time dependent with complete inactivation in 10 min at 0.3 μM ODQ (Schrammel *et al*., 1996). ODQ is used increasingly as a pharmacological tool for discrimination between cGMP dependent and independent actions of NO. So our present study is to focus on whether insulin mediated relaxation through activation of sGC or not.

MATERIALS AND METHODS

Drugs and chemicals

1H-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one (ODQ), (Cayman Chemical, USA); 4-Aminopyridine (4-AP), L-arginine (HiMedia, India); Acetylcholine chloride (ACh), Noradrenaline (NA), Phenylephrine hydrochloride (PE) (Sigma, USA) were the drugs employed for the study.

Collection and preparation of GIIA

Goat internal iliac artery was collected from local abattoir within 20-30 min of slaughter in cold, aerated Modified Kreb's Henseleit solution (MKHS). The right internal iliac artery branches were carefully dissected out into a petridish containing aerated physiological solution. Each artery was cleaned off its fat and connective tissue and was cut into rings of 2-2.5 mm length and 2-3 mm external diameter.

Vascular tension study

Tissue was mounted on automatic organ bath following the similar procedure as described earlier by us (Arunvikram *et al*., 2015). After equilibrating for 90 min, internal iliac artery rings were contracted with PE / 5-HT. At the plateau of PE / 5-HT with sustained contraction, ACh (1.0 μM) was added to determine endothelial integrity. A relaxant (70 %) response to ACh confirmed the presence of a functional endothelium, while absence of appreciable relaxation (10 %) after mechanical denudation confirmed satisfactory endothelium removal. The preparation was

Table 1:
pD_2 and E_{max} of PE (10^{-5} M) induced contraction and relaxation by ACh in presence of insulin or insulin+ ODQ or insulin+ODQ +4-AP.

Treatment	n	Concentration(M)	pD_2		E_{max}/E_{Bmax} (%)	
			Mean	SEM	Mean	SEM
ACh(CONTROL)	6		6.6	0.19	72.9	2.07
INS+ODQ	6	1μM+10μM	6.37	0.53	24.45***	3.25
INS+ODQ+4-AP	6	1μM+10μM+500μM	7.73**	0.26	23.69***	4.96
INS	6	1μM	7.66*	0.17	78.2	8.6

* Values differ significantly when compared with control (*P<0.05, **P<0.01 and *** P<0.001)

Table 2:
pD_2 and E_{max} of 5-HT (10^{-5} M) induced contraction and relaxation by ACh in presence of insulin or insulin+ODQ or insulin+ODQ+4-AP

Treatment	n	concentration(molar)	pD_2		E_{max}/E_{Bmax} (%)	
			MEAN	SEM	MEAN	SEM
ACh(CONTROL)	6		5.8	0.25	76.91	1.28
INS+ODQ	6	1μM+10μM	8.36*	0.85	24.71***	2.78
INS+ODQ+4-AP	6	1μM+10μM+500μM	7.57*	0.43	24.55***	2.11
INS	6	1μM	6.41	0.26	82.79	4.68

*Values differ significantly when compared with control (*P<0.05, **P<0.01 and ***P<0.001)

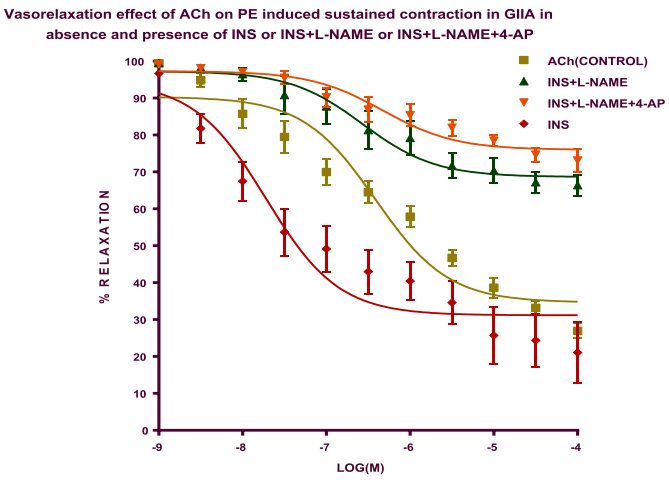

Fig 1:
Effect of PE (10^{-5} M), induced contraction and relaxation by ACh in presence of insulin or insulin+ODQ or insulin+ODQ+4AP

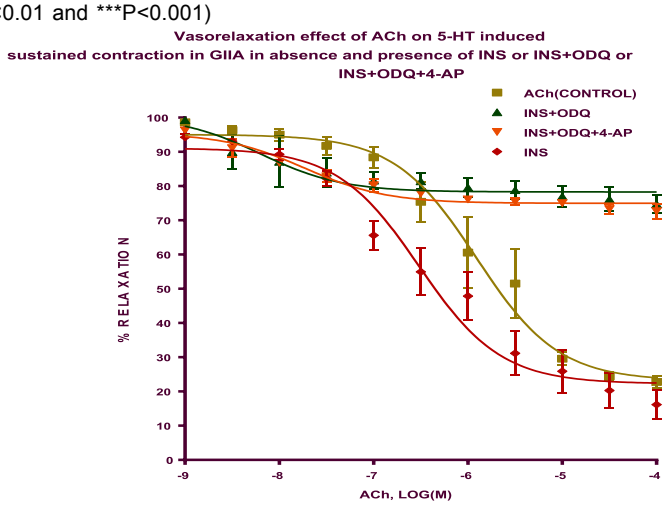

Fig. 2:
Effect of 5-HT (10^{-5} M), induced contraction and relaxation by ACh in presence of insulin or insulin+ODQ or insulin+ODQ+4AP

washed with MKHS solution to restore baseline resting tension.

Experimental design

Effect of PE (10^{-5} M) and 5-HT (10^{-5} M) induced contraction and relaxation by ACh in presence of insulin or insulin+ODQ or insulin+ODQ+4AP

Before PE(10^{-5} M) or 5-HT(10^{-5} M) induced contraction, GIIA rings were pre-incubated for 30 min with insulin(10^{-6}M)+ODQ(10^{-5}M) or insulin(10^{-6}M)+ODQ(10^{-5} M)+4AP(10^{-5} M) or insulin(10^{-6}M). After attainment of sustained contraction with PE / 5-HT (10^{-5} M), ACh (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M and 10^{-4} M) was added in a cumulative manner with 0.5 log unit increment at 4min interval. The pD_2 and E_{Bmax} were recorded for each experiment and compared.

RESULTS

Effect of PE (10^{-5} M) induced contraction and relaxation by ACh in presence of Insulin or Insulin+ODQ or Insulin+ODQ+4AP

The relaxant response of ACh was significantly inhibited with a increase in mean pD_2 and decrease in maximal response in presence of INS+ODQ+4-AP (pD_2 7.73 ± 0.26, p<0.01 and E_{Bmax} 23.69 ± 4.96 %, p<0.001) whereas, decrease in mean pD_2 and maximal response in presence of INS+ODQ (pD_2 6.37 ± 0.53 and E_{Bmax} 24.45 ± 3.25 %, p<0.001). In presence of insulin, the vasorelaxation of ACh was not significantly increased with increase in pD_2 (7.66 ± 0.17 Vs 6.6 ± 0.19, p<0.05) and E_{Bmax} (78.2 ± 8.6 % Vs 72.9 ± 2.07 %). In conclusion, Insulin increased the affinity and slightly increased the vasorelaxation effect of ACh in PE pre-contracted GIIA rings (Table 1 and Fig 1).

The relaxant response of ACh was significantly inhibited with an increased in mean pD_2 and decreased in maximal response in presence of INS+ODQ (pD_2 8.36 ±

0.85, p<0.05 and E_{Bmax} 24.71 ± 2.78 %, p<0.001) and INS+ODQ+4-AP (pD_2 7.57 ± 0.43, p<0.05 and E_{Bmax} 24.55 ± 2.11 %, p<0.001). In presence of insulin, the vasorelaxation of ACh was not significantly increased (p<0.05) with increase in pD_2 (6.41 ± 0.26 Vs 5.8 ± 0.25) and E_{Bmax} (82.79 ± 4.68 % Vs 76.91 ± 1.28 %). In conclusion, insulin slightly increased the affinity and increased the vasorelaxation effect of ACh in 5-HT pre-contracted GIIA rings (Table 2 and Fig 2).

DISCUSSION

In the present study, in ACh induced endothelium-dependent relaxation, pretreatment of INS+ODQ caused a remarkable decrease in the E_{max} from control in about PE (48 %) and 5-HT (52 %) pre-contracted tissue. Likewise, pretreatment of INS+ODQ+4-AP, decrease in the E_{max} from control was in about PE (49 %) and 5-HT (52 %) pre-contracted tissue. These findings suggest that INS+ODQ as well as INS+ODQ+4-AP inhibited the ACh induced vasorelaxation. It was found that inhibitory effect of INS+ODQ and INS+ODQ+4-AP was most prominent in ACh induced vasorelaxation in NA pre-contracted GIIA rings. There was no significant change in relaxation response of ACh, while using 4-AP along with INS+ODQ, there was no synergistic or antagonistic effect as compared to INS+ODQ alone. Thus it indicates that voltage gated K^+ channels are not much involved in insulin mediated relaxation of goat internal iliac artery.

The are several reports convinced that vasorelaxation effects exerted by insulin on vascular smooth muscle cells may be attributable to an increase of both cAMP and cGMP through receptor-mediated activation of adenylate and of guanylate cyclases. Thus, the effect of insulin on cGMP is mediated by nitric oxide (Trovati *et al.,* 1995). These provide potential mechanisms for a decrease in vascular smooth muscle tone by insulin and for diminished responsiveness to vasoconstrictor agents. It has been suggested that insulin's vasodilatory action may be closely associated with an endothelium-dependent vasorelaxant mechanism. In this study, ACh induced endothelium-dependent relaxation, pretreatment of insulin did not cause a remarkable increase in the E_{max} from control in about in PE (5 %) and 5-HT (6 %) pre-contracted tissues. These findings suggest that insulin enhances the vascular relaxation might be due to sGC dependent pathway in GIIA. However, further studies on molecular level are required for characterizing the various receptors and their subtypes responsible for this mechanism and signaling pathways in GIIA.

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Research Article

PHARMACOKINETICS OF AMOXICILLIN FOLLOWING ORAL ADMINISTRATION IN CALVES

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ABSTRACT

The present study on pharmacokinetics of amoxicillin in calves was carried out in six apparently healthy male calves (2-3 months of age). Amoxicillin was given at dose rate of 10 mg.kg^{-1} by oral route. The blood samples were collected at 15 min., 30 min., 1, 2, 3,4,5,6,8,10 and 12h. after oral administration. The samples were analysed for amoxicillin using agar plate diffusion method. The disposition pattern followed two compartment open model. After oral administration the peak plasma level was obtained at 3 h and the values of AUC, $t_{1/2\beta}$, Cl_B and MRT were: 9.25 ± 0.31 µg.ml^{-1}.h, 1.66 ± 0.05 h, 617.03 ± 10.71 ml.Kg^{-1}.h^{-1} and 3.79 ± 0.06 h, respectively. The pharmacokinetic parameters of the distribution and elimination phase of amoxicillin revealed rapid distribution of the drug into body fluids.

Key words: Pharmacokinetics, Amoxicillin, Agar plate diffusion method

INTRODUCTION

Pharmacokinetics is a mathematical description of concentration changes of drug as function of time in the body and includes the study of drug absorption, distribution, metabolism, and excretion. The microbial infections are major causes of animal diseases and therefore, antimicrobials are amongst the most commonly used of all drugs. Amoxicillin, an acid stable, semi-synthetic drug shown to be effective against a wide range of infections caused by wide range of gram- positive and gram-negative bacteria in both human and animals (Kaur *et al*., 2011). Majority of bacteria like *Escherichia coli, Salmonella pullorum, Salmonella gallinarum, Salmonella enteritidis, Proteus vulgaris, Klebsiella pneumonia, Citrobacter freundii, Clostridium perfringes and Corynebacteium pyogenes* are sensitive to amoxicillin (Hooda *et al*., 2011). The most frequently used combination in veterinary practice is amoxicillin-clavulanic acid and is preferred due to its broad antimicrobial activity, bactericidal effects, and high therapeutic index (Jerzsele and Semjen, 2006). Pharmacokinetic studies injection of amoxicillin have been reported in calves (Nouws *et al*., 1986) in pigs (Larranaga *et al*., 2004), in sheep goats (Craigmill *et al*., 1992). and horses (Ensink *et al*., 2008). Comparative pharmacokinetics and dosage regimen of amoxicillin in febrile dog after oral suspension of amoxicillin (20 mg.kg^{-1}) have been reported (Marrier *et al*., 2002). Calf diarrhea is the leading cause of mortality in dairy calves. Calves with diarrhea often have small intestinal overgrowth with *E.coli* bacteria, regardless of the inciting cause for the diarrhea, and 30% of systemically ill calves with diarrhea have bacteremia, predominantly because of *E.coli.* (Constable, 2004). The present study was designed to investigate the pharmacokinetics of amoxicillin after oral administration in calves.

MATERIALS AND METHODS

The present study was conducted in 6 apparently healthy male cow calves of 2-3 months age. The animals were kept in the experimental shed of COVAS, CSKHPKV, Palampur and were provided Calf growing ration, green fodder and water *ad lib*. Amoxicillin pure (Hi Media, Mumbai, India.) was administered orally (10 mg/kg, bw) to these calves. Blood samples were collected from jugular vein in heparinised vials at 15 min, 30min, 1, 2, 3, 4, 5, 6, 8, 10 and 12h. The plasma was separated after centrifugation at 3000 rpm for 15 min. at room temperature and stored at -20º C until further analysis. The concentration of amoxicillin in plasma was determined by using modified agar plate diffusion method (Bennet *et al*., 1966) with *Escherichia coli* (MTCC 443) used as a test organism growing on Muller Hinton Agar (Hi Media Laboratories, Mumbai India). Six wells, 8.0mm in diameter, were cut at equal distances into a standard petri plate containing 12ml of the seeded agar. One well on each plate was filled with reference concentration (0.6 µg.ml^{-1}) and the remaining wells were filled with 50µl of the samples. The plates were kept at room temperature for 4 hours and then incubated for 14-16 h at 37º C. At the end of incubation, diameter of each zone of inhibition was measured using vernier callipers and the values for zone of inhibition were obtained. The values of amoxicillin were expressed in terms of µg.ml^{-1} in plasma.

Pharmacokinetic analysis

The plasma concentration-time profile of amoxicillin of each animal was used to determine the pharmacokinetic variables. The pharmacokinetic data of amoxicillin was subjected to compartmental analysis. The standard curve was plotted using different concentrations by microbiological assay.The pharmacokinetic parameters

were analyzed according to the method of Gibaldi and Perrier (1982). The data was analyzed by analysis of variance test using the Graph Pad Instat version 3.00 for windows (GraphPad Software, SanDiego, California, USA, (www.Graphpad.com)

RESULTS

Mean (± SEM) plasma amoxicillin concentrations after oral administration (10mg/kg) in cow calves are shown in Fig.1. The plasma concentration of amoxicillin was 0.49 ± 0.02 µg.ml^{-1} at 30 min. The peak plasma concentration of amoxicillin (2.40 ± 0.08 µg.ml^{-1}) was achieved at 3 h, which gradually declined to 0.20 ± 0.01 µg.ml^{-1} at 8 h and 0.07 ± 0.01 µg.ml^{-1} at 12 h. The evaluation of observed plasma levels of amoxicillin indicated that the drug data could be best fitted to a two compartment open model. Various pharmacokinetic variable, which describe the distribution and elimination of amoxicillin in calves are listed in Table 1. The values of area under the plasma distribution time curve (AUC), elimination half life ($t_{1/2\beta}$),total body clearance(Cl_B), Mean residence time (MRT) were: 9.25 ± 0.31 µg.ml^{-1}.h, 1.66 ± 0.05 h, 617.03 ± 10.71 ml.Kg^{-1}.h^{-1} and 3.79 ± 0.06 h, respectively.

Table1:

Pharmacokinetic parameters (mean± SE) of amoxicillin following single oral administration @ 10mg.kg^{-1} body weight in cow calves.

Pharmacokinetic parameters	Units	Mean± SE
A2	µg.ml^{-1}	9.23 ± 0.59
B	µg.ml^{-1}	7.94 ± 0.56
K_a	h^{-1}	0.96 ± 0.03
B	h^{-1}	0.42 ± 0.01
$t_{1/2\,Ka}$	H	0.72 ± 0.02
$t_{1/2\,\beta}$	H	1.66 ± 0.05
AUC	µg.ml^{-1}.h	9.25 ± 0.31
AUMC	µg.ml^{-1}.h^2	35.03 ± 1.37
MRT	H	3.79 ± 0.06
C_{max}	µg.ml^{-1}	2.4 ± 0.08
Cl_B	ml.kg^{-1}.h^{-1}	617.03 ± 10.71
$V_{d\,(area)}$	l.kg^{-1}	1.48 ± 0.06
t_d	H	6.63 ± 0.20
F	Fraction	0.57 ± 0.02

$t_{1/2\alpha=}$ Distribution half life; AUC= Total area under the plasma conc. Curve; AUMC= Total area under the first moment of plasma drug concentration-time curve; $V_{d(area)}$=Apparent volume of distribution; B= zero time intercept of elimination phase; $t_{1/2\,\beta=}$ Elimination half life; Cl_B =Total body clearance; MRT=Mean residence time. F= Bioavailability of drg ,C_{max}: peak plasma drug concentration, t_d :Total duration of pharmacological effect , $t_{1/2Ka:}$ Absorption half life , K_a :rate constant of absorption of drug after oral administration. A^1: zero time drug plasma concentration intercept of regression line of absorption phase. β : overall elimination rate constant

DISCUSSION

The plasma concentration of amoxicillin was 0.49 ± 0.02 µg.ml^{-1} at 30 min. The peak plasma concentration of amoxicillin (2.40 ± 0.08 µg.ml^{-1}) was achieved at 3 h, which gradually declined to 0.20 ± 0.01 µg.ml^{-1} at 8 h and 0.07 ± 0.01 µg.ml^{-1} at 12 h. similar levels in peak serum concentration have been reported after single oral administration in calves (Palmer *et al*., 1977). The plasma drug concentration versus time curve of the drug following oral administration of ampicillin was best described by two compartment model (Black, 1976; Mercer *et al*., 1977; Galtier and Charpentean 1979).The evaluation of observed plasma levels of amoxicillin indicated that the drug data could be best fitted to a two compartment open model and described in terms of bi-exponential equation as:

$$C_p^{(t)} = Ae^{-\alpha t} + Be^{-\beta t}$$

Where, $C_p^{(t)}$ is the concentration of drug in plasma at time t, A^1 and B are zero time plasma drug concentration intercepts respectively for absorption and elimination components. K_a and β are the first order rate constants for the absorption and elimination phase, respectively and e is the base of natural logarithm. The mean value of absorption rate constant K_a and absorption half-life were 0.96± 0.03 h^{-1} and 0.72 ± 0.02 h, respectively. The absorption half-life of amoxicillin observed in the present investigation was almost identical to that of humans (Weber *et al*., 1984).Various other coefficients of elimination and absorption phases were: B (7.94 ± 0.56 µg.ml^{-1}), β (0.42 ± 0.01 h^{-1}), A^1 (9.23 ± 0.59 µg.ml^{-1}) and K_a (0.96 ± 0.03 h^{-1}). The mean plasma half-life of amoxicillin (0.5 to 1.2 h) in other domestic species was similar to the present investigation (Giguere *et al*., 2013) and mean plasma concentrations were achieved almost in identical time in calves (Musser and Anderson, 2001).The value of overall elimination rate constant (β) was 0.42 ± 0.01 h^{-1} whereas, the values of elimination half-life ($t_{1/2\beta}$) and area under the curve (AUC) were: 1.66 ± 0.05 h and 9.25 ± 0.31 µg.ml^{-1}.h, respectively. However AUC reported by Musser and Anderson (2001) was much lower than those observed in the present study. The bioavailability is a better indicator of the therapeutic efficacy of the drug. A higher drug bioavailability is pertinent for a successful drug therapy. The bioavailability of 0.57 in the present study indicated that the drug can be successfully used after oral

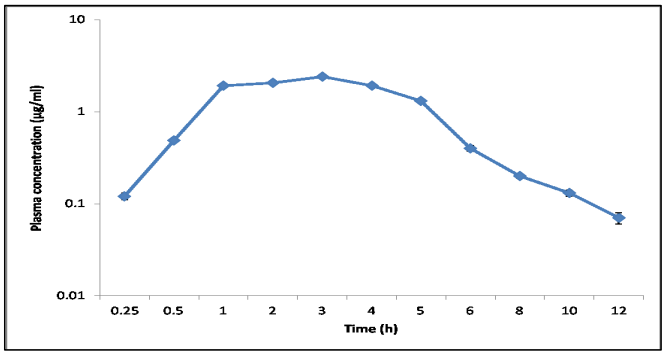

Fig 1:

Semilogarithmic plot of the mean plasma concentration-time profile following oral administration (10mg/kg b.w) in cow calves. Values presented are mean±SE of six animals.

administration in calves. The bioavailability of amoxicillin observed in the present study was quite closer to the bioavailability of ampicillin in ruminant calves as recorded in an earlier study (Nouws *et al*., 1982). The mean residence time (MRT) observed after oral administration of amoxicillin was greater in the present study indicating that the drug is persistent for a long time after oral administration. Following oral administration the pharmacokinetic profile of amoxicillin was found to be favourable. The use of amoxicillin (10mg/kg) might be clinical alternative for treatment of most bacterial infections in calves.

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Research Article

EVALUATION OF ANTIOXIDANT EFFICACY OF COW URINE DISTILLATE IN IMIDACLOPRID INTOXICATED WLH COCKERELS

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ABSTRACT

Imidacloprid, a chloronicotyl was the first representative of neonicotinoids insecticides to be registered for use. The present study was aimed towards the evaluation of antioxidative effect of cow urine distillate following administration of imidacloprid in white leghorn cockerels for 60 days. Forty eight birds were divided into six different groups randomly and equally. Group I served as control, whereas, group II, III, V and VI were given imdacloprid in feed @ 14.1 and 7.05 mg/ kg (1/ 10^{th} and $1/20^{th}$ of LD_{50}) and groups V and VI were also given cow urine distillate orally @ 1ml/bird daily for 60 days. Group IV was given only cow urine distillate @ 1ml/bird/day for 60 days. Levels of LPO, GSH and catalase activity were measured in liver tissues by using standard protocols. The study concluded that imidacloprid produced the dose dependent oxidative stress in white leghorn cockerels and the cow urine distillate has showed the antioxidative potential as the changes in treatment group were less significant as compared to the intoxicated group.

Key Words: Imidacloprid, Cow urine distillate, LPO, GSH, Catalase, White leghorn cockerels

INTRODUCTION

To fulfil the needs of large number of people, indiscrimate and injudicious use of insecticides are done by the agricultural farmers. Insecticides are divided into various classes on the basis their chemical nature. The extensive use of pesticide in agricultural land also compromising the soil and water quality of the land and residues of pesticide used may remain above the maximal residual levels resulted into serious health hazards (Yassi *et. al*., 2001). These problems are being seriously surveillance by the international agencies as United States Environmental Protection Agency (USEPA), Codex Alimenterious Commission, World Health Organization (WHO) and FAO of United Nations (Cao *et. al.,* 2005). Imidacloprid, a chloronicotyl was the first representative of neonicotinoids insecticides to be registered for use and currently the most important commercial product because of its high efficacy against insects and low soil persistence (Chao and Casida, 1997). According to the report of Buckley *et al.,* (2004), there was three million cases of severe poisoning and 2, 20,000 deaths Indian subcontinents especially from the Asian countries. Currently, India is the largest producer of pesticides in Asia and ranks twelfth in the world for the use of pesticides (Gunnell and Eddleston, 2003). The report was indicator of indiscriminate use of pesticides in the agricultural fields. The current neonicotinoids and their year of patents are as follows: Imidacloprid (1985), thiacloprid (1985), thiamethoxam (1992), acyclic nitenpyram (1988), acetamiprid (1989), clothianidin (1989) and dinotefuran (1994) (Tomizawa and Casida, 2005).

Poultry industry is one of the fastest growing industries among the livestock sector. The normal physiology must have to be maintained in order to produce the maximum amount of meat and quality eggs from the poultry. Imidacloprid produces thinning of egg shell, and reduced egg hatchability which are indicators of the endocrine disruption potential of imidacloprid (Berny *et al*., 1999 and Matsuda *et al.,* 2001). It is a systemic insecticide that translocate rapidly through plant tissues following application (Tomlin, 2006 and Fossen, 2006). Panchgavya has been mentioned in ancient ayurvedic literature as one of the most potent remedies for the deadly disease if taken on daily basis. It had been proven that cow urine distillate had immunomodulatory action and anti oxidative potential (Bapu, 2001). Chauhan *et. al.,* 2009 has also reported that the alternative kind of therapy termed as 'cowpathy', has been reported to be beneficial even for dreaded diseases like cancer, AIDS and diabetes In Sushrita Samhita the cow urine has been described as the most effective substance of animal origin with innumerable therapeutic values (Dhama *et al.,* 2005). Thus in this context the aim of the study was to assess the antioxidative potential of cow urine distillate following sub chronic to leghorn cockerels.

MATERIALS AND METHODS

The present study was conducted in 48 white leghorn cockerels, procured from IPF, GBPUAT, Pantnagar. These birds were acclimatized for two weeks in the experimental animal shed of Instructional poultry farm, Nagla, Pantnagar under standard managemental conditions. Poultry feed and water were provided *ad libitum* and kept under constant observation throughout study. All the chemicals required for this study were procured from Hi Media. Initially 48 birds were divided randomly and

Table 1: *In vivo* 60 days sub chronic toxicity study

Group	Treatment	Dose	Route	Period of exposure
I	Control untreated	-	-	-
II	Imidacloprid	1/10th LD_{50}	Oral	60 days
III	Imidacloprid	1/20th LD_{50}	Oral	60 days
IV	Control treated with cow urine	1ml/bird/day	Oral	60 days
V	Imidacloprid + Cow urine (I)	1/10th LD_{50}Imidacloprid + cow urine @ 1ml/bird/day	Oral	60 days
VI	Imidacloprid + Cow urine (II)	1/20th LD_{50}+ Cow urine @ 1ml/bird/day	Oral	60 days

Table2:Effect of imidacloprid on LPO (nM MDA/ml) , GSH (µM/ml) and catalase activity in liver of white leghorn cockerels (Mean ± S.E., n=8).

Groups /Days	LPO (nM MDA/ml)	GSH (µM/ml)	Catalase activity (nmol/min/mgprotein)
Group I	0.531±0.017^{B}	2.896±0.028^{E}	8136.78±154.41^{D}
Group II	0.900±0.017^{F}	1.541±0.007^{AB}	1609.76± 132.54^{AB}
Group III	0.813±0.020^{E}	1.506±0.013^{A}	2254.56± 122.78^{A}
Group IV	0.483±0.013^{A}	2.561±0.033^{D}	8013.16±134.32^{D}
Group V	0.756±0.015^{D}	1.620±0.047^{B}	3299.893±186.43^{B}
Group VI	0.693±0.011^{C}	1.911±0.042^{C}	4699.893±186.43^{C}

Mean value bearing capital alphabets differ significantly (P<0.05) when compared vertically with in the same column.

equally in six different groups having eight (n=8) in each group. Group I was kept as control, group II and III were provided with imidacloprid @ 1/10th and 1/20th of LD_{50} respectively and Group IV was given only cow urine distillate @1 ml/bird/day, whereas group V and VI were given imidacloprid @1/10th and 1/20th of LD_{50} dose with cow urine distillate treatment @1 ml/bird/day, respectively for a period of sixty days continuously by oral dosing (Table 1). At the end of experimental period the birds were humanely sacrificed and liver and kidney were collected to assess the oxidative stress parameters as LPO, GSH and catalase by the standard protocols. Experiment was conducted after the permission of the Institute animal ethics committee (IAEC) and adequate measures were taken to minimize pain or discomfort to animals.

Statistical analysis

Statistical analysis of data was done by using ANOVA technique. Comparisons among treated and untreated groups were made with help of student't' test. Statistically significant difference was considered at 5 and 1 percent level (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

The present study revealed a significant increase in LPO values in liver tissues of imidacloprid treated groups II, III, V and VI as compared to the control group I and group IV as evidenced by the increased production of malondialdehyde (MDA) after 60 days post exposure of toxicant (Table 2). A non significant change was observed in between group IV and control group I. Insecticides may induce the oxidative stress, leads to generation of free radicals and alteration in antioxidants, oxygen free radicals, scavenging enzyme system and lipid peroxidation thus contributes to toxicity (Abdollahi *et al.,* 2004; Broznic *et al*., 2008). These elevated levels of free radicals and ROS may lead to disruption of cell membrane, oxidative damage to cell membrane and hence increase susceptibility to LPO (Kapoor *et al.,* 2009). The damage of membrane lipids, DNA and protein molecules are the endpoint markers of oxidative stress inducing toxic effect of pesticides. The study was in agreement with the findings of Jiao-Jiao *et al*., (2011), who showed similar observation following oral administration of acetamiprid in male mice. There was significant (P<0.05) decrease in GSH levels and catalase activity in liver tissues in groups II, III, V and VI as compared to control groups I and IV after 60 days post exposure of toxicant (Table 2). A non significant change was observed in between group IV and I. GSH plays an important role in antagonizing the oxidative action of the herbicides or insecticides (Parke and Piotrowski, 1996). Hepatic GSH plays a crucial role in both scavenging reactive oxygen species and the detoxification of xenobiotics (Haque *et al.,* 2003). Catalase is a unique enzyme that can remove H_2O_2 present in high concentrations. The decreased values of LPO and increased value of GSH and Catalase activity in group IV, V and VI showed the antioxidative potential of cow urine distillate on regular use basis.

It is concluded from the study that imidacloprid causing oxidative damage to the liver in a dose dependent manner following sub chronic exposure to white leghorn cockerels and the cow urine distillate has produced significant anti oxidative effect by modulating the oxidative stress markers as LPO, GSH and catalase activity in liver tissues. Study is an indicative of the fact that the regular use of cow urine distillate will be helpful in removing the free radical and other reactive oxygen species thus modifying the health complacencies and improve health status.

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Research Article

COMPARISON OF PESTICIDE RESIDUES IN BUFFALO PLASMA COLLECTED FROM TWO DIFFERENT DISTRICTS OF PUNJAB

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ABSTRACT

Pesticides are most commonly used by farming communities. In Punjab against various pests during various agricultural operations. The survey was done in two districts of Punjab viz Ludhiana, and Sangrur. In each district some villages were randomly selected and from each village samples of buffalo blood were collected. The comparison of current levels of pesticide residues in ninety five samples of buffalo blood collected from two different districts of Punjab by using Gas chromatography was done. Residues of a-HCH, β-HCH, γ-HCH, δ-HCH, heptachlor, fenitrothion, aldrin, fipronil, butachlor, dieldrin, p,p-DDE, o,p-DDE, p,p-DDT, o,p- DDT, endosulfan, L- cyhalothrin, cyfluthrin, cypermethrin , fenvalerate and deltamethrin were found in buffalo blood samples. L-cyhalothrin had highest mean residue concentration in the two districts, while endosulphan, heptachlor and aldrin had lowest mean residue level. Percentage contribution of α- HCH and δ-HCH was highest in Sangrur district. The detection of OCPs and their degradation products in air, water, soil and sediments, fish, birds and food stuffs has become a matter of great concern posing threat to animal and human health. The residue monitoring studies in blood may be investigated further in order to improve food safety.

Key Words: Pesticide, Punjab, Gas chromatography.

INTRODUCTION

India is home to approximately 16% of the total world's population, but has just less than 2% of the total landmass. Rapid population growth, together with a high emphasis on achieving food grain self-sufficiency has compelled Indian farmers to resort to the substantial use of pesticides Among the various states of India, Punjab was the hub of the Green Revolution, which made the Punjab's farmers self-sufficient, no longer dependent on other states and countries for their basic needs of food (Sidhu and Byerlee 1992). As per the *Statistical Abstract of Punjab* (2005) report, the grain production in Punjab increased from 3.16 million tons in 1960–1961 to 25.66 million tons in 2004–2005. This increased production had some major drawbacks, one of which was high use of pesticides (Yadav 2006). The abundant use of pesticides constitutes a danger not only for aquatic and terrestrial biodiversity but also for humans because of their presence in food chain. The environmental conditions in tropical countries are highly conducive to rapid multiplication of pests. Therefore, a wide variety of pesticides is used in tropical countries to combat these crop pests and disease vectors. However, some characteristics, such as persistency, volatility and atmospheric distribution through long range transportation has resulted in the contamination of air, water, soil and food (Kim and Smith, 2001; Singh *et al*., 2005).

MATERIALS AND METHODS

The survey was done in two districts of Punjab viz Ludhiana, and Sangrur. In each district some villages were randomly selected and from each village samples of buffalo blood were collected. From Ludhiana district forty five samples and from Sangrur district fifty blood samples were collected. Therefore a total of 95 samples of buffalo blood were collected from the two districts. Venous blood (5 ml) was collected in residue free heparinised vials containing 200 USP units of heparin in 0.2 ml solution with the help of sterilized syringe. Blood samples were transported in dry ice in the laboratory and stored at -20^{0}C until analyzed. A questionnaire was designed to collect the information regarding the animal and analysis of pesticide residues was done in laboratory. Blood samples were centrifuged at 3000rpm for 15 min to obtain plasma. Extraction was done by the method given by Gill *et al* 1996 with slight modifications. The plasma samples were thawed and vortexed. An aliquot (1ml) was placed into a centrifuged tube. After equilibration at room temperature for 15 min, acetic acid (1 ml) was added and the tube was vortexed for 1 min. The analytes were extracted with hexane/ DCM (9:1 v/v, 3 ml); vortexed for 1 min and centrifugation was done at 1800 rpm for 2 min. The top organic layer was drawn into a clean centrifuge tube. The extraction was repeated twice with hexane/ DCM with 3ml amount each time .The combined organic phase was concentrated to 0.5 ml using rotary vacuum evaporator for clean up.

Clean up was done by USEPA method 3620B (USEPA 2003) – Florisil clean up by column chromatography. Florisil was activated at 130^{0}C overnight and cooled in a dessicator before use. One gram florisil

was packed in the glass chromatographic column in between two layers of anhydrous sodium sulfate (0.5 g). The column was pre-eluted with hexane and discarded. Then the concentrated sample extract was added to the column. The extract tube was rinsed twice with 0.5 ml of hexane. For recovering organochlorines and synthetic pyrethroids, elution was carried out with 8 ml hexane. The resultant elute was concentrated to 2-5 ml and stored for analysis in Gas Chromatograph (Perkin Elmer Clarus 500).The Electron Capture Detector (ECD) was used for estimation of OCPs and synthetic pyrethroids (SPs). The compounds were identified and quantified by comparison of the retention time and peak heights/area of the sample chromatograph with those of standards run under the same operating conditions. Reagent blank samples were run every six samples to check the interference or contamination from solvents and glassware. The limit of detection ranged from 1.0 ng g^{-1} for OCPs and SPs.

Statistical analysis

Statistical analysis was carried out using the SPSS Microsoft version 16.0 for windows. Residue levels of pesticides in buffalo blood were summarized using arithmetic means, standard deviations together with minimum and maximum values. The samples negative for pesticide residues were considered as not detected (ND) and treated as zero in calculations.

RESULTS AND DISCUSSION

In this study residues of α-HCH, β-HCH, γ-HCH, δ-HCH , Heptachlor, Fenitrothion, Aldrin, Fipronil, Butachlor, Dieldrin, p,p- DDE, o,p- DDE, p,p- DDT, o,p- DDT ,endosulfan, L- cyhalothrin, cyfluthrin, cypermethrin , fenvalerate and deltamethrin were found in buffalo blood samples(Table 1 and 2). L- cyhalothrin had highest mean

Table 1: Levels of pesticide residues (ng ml-1) in buffalo blood samples from Ludhaina district of Punjab.

S.No	Pesticides	Mean	SE	Median	Range
1	α-HCH	0.2778	0.0844	0.2418	ND-0.3842
2	γ-HCH	0.1060	0.0220	0.1185	ND-0.1218
3	δ-HCH	1.0375	-	1.037	-
4	Heptachlor	1.907	0.3324	1.635	ND-6.929
5	Fenitrothion	2.282	1.022	2.052	ND-4.140
6	Aldrin	0.1658	0.0493	0.1132	ND-0.4316
7	Fipronil	1.297	0.5431	0.9953	ND-2.571
8	Butachlor	1.406	0.4676	1.111	ND-1.537
9	Dieldrin	0.1990	-	0.1991	-
10	p,p- DDE	1.270	-	1.270	-
11	o,p DDT	1.097	0.6995	1.097	ND-1.399
12	Endosulphan- SO4	0.0228	-	0.0228	-
13	p,p-DDT	0.2010	-	0.2010	-
14	L- Cyhalothrin	3.828	1.654	1.121	ND-20.59
15	Permethrin	0.8917	0.1640	0.8917	ND-0.3281
16	Cyfluthrin	0.5023	0.3227	0.5023	ND-0.6454
17	Cypermethrin	0.3946	0.1642	0.3946	ND-0.3284
18	Fenvalerate	2.434	0.8310	1.307	ND-6.185
19	Deltamethrin	2.456	0.0393	2.456	ND-0.0786

Table 2: Levels of pesticide residues (ng ml^{-1}) in buffalo blood samples from Sangrur district of Punjab.

S.No	Pesticides	Mean	SE	Median	Range
1	α-HCH	0.3348	0.0403	0.31255	ND- 0.7749
2	β-HCH	0.1163	0.0368	0.08990	ND-0.1566
3	γ-HCH	0.408	0.1741	0.1729	ND-3.787
4	δ-HCH	1.47	0.7522	0.8051	ND-2.338
5	Heptachlor	0.02	0.02	1.654	ND-9.019
6	Fenitrothion	3.25	1.3682	2.479	ND-18.80
7	Aldrin	0.1541	0.0303	0.1209	ND-0.4065
8	Fipronil	1.08	0.3053	0.87910	ND-3.910
9	Butachlor	0.7057	0.1972	0.6770	ND-0.6816
10	Dieldrin	1.9	0.3461	1.463	ND-10.47
11	p,p- DDE	0.2967	0.04004	0.2589	ND-.02610
12	o,p- DDE	0.2008	-	0.2008	ND-0.0000
13	o,p DDT	0.1583	-	0.1583	ND-0.0000
14	Endosulphan- SO_4	0.5937	0.20845	0.5937	ND-0.4169
15	p,p-DDT	0.24	23.815	0.2489	ND-95.38
16	L- Cyhalothrin	3.95	0.5657	2.970	ND-14.39
17	Cyfluthrin	0.843	79.2917	2.468	ND-2.779
18	Cypermethrin	1.21	0.2	0.7376	ND-4.582
19	Fenvalerate	2.14	0.3862	1.5120	ND-7.832

residue concentration in the districts, while Endosulphan, Heptachlor and aldrin had lowest mean residue level. HCH residues were detected, in all the two districts. Percentage contribution of α- HCH and δ-HCH was highest in Sangrur district. Since 1996, DDT was banned as an agricultural pesticide (Battu *et al.* 2004); however, nearly 85% of the DDT produced in India is used for public health practices for residual spray (Sharma 2003). The possible sources of DDTs are the combined effect of past and ongoing use in vector control or from sprays on open dumping sites. Among DDT metabolites, p,pDDE was found at highest mean concentration in Ludhiana district followed by o,p-DDT and p,p- DDT. While in Sangrur district all the metabolites of DDT were present in lower concentrations. The pesticide residues of Hepachlor, Fenitrothion, Aldrin, fipronil, butachlor, dieldrin, endosulphan , L- cyhalothrin, cyfluthrin, cypermethrin and fenvalerate were present in all the two districts. Deltamethrin was present in Ludhiana district only. The predominant pesticide residues in Ludhiana district are of Heptachlor followed by L-Cyhalothrin, Aldrin and fenvalerate. In Sangrur district Heptachlor was leading followed by cyfluthrin, dieldrin , L- cyhalothrin and cypermethrin.

It could be concluded that OC pesticide residues were detected in buffalo's blood as they were persistent in nature due to their slow decomposition rate, long half-life and high stability in the environment. Nevertheless, the residue monitoring studies in blood should be investigated further in order to improve food safety since these compounds represent a potential risk to human health.

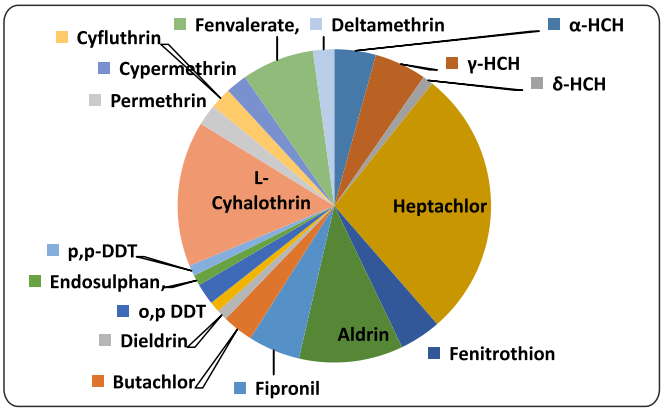

Fig 1:
Distribution of pesticide residues (%) in the buffalo blood samples from Ludhaina district of Punjab.

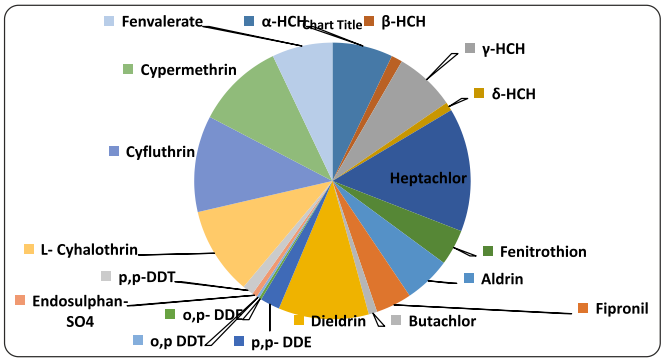

Fig. 2:
Distribution of pesticide residues (%) in the buffalo blood samples from Sangrur district of Punjab.

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Research Article

TOXICODYNAMIC INTERACTIONS OF IMIDACLOPRID IN MICE: AN INSIGHT INTO ITS MECHANISM OF ACTION

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ABSTRACT

Imidacloprid potentiated the experimentally induced convulsions to the highest degree i.e. tonic convulsions or death in all the models (maximal electroshock seizures, pentylenetetrazole, strychnine, tremorine, picrotoxin, and phenytoin-pentylenetetrazole) except status epilepticus induced by lithium pilocarpine. The effects of imidacloprid on experimentally induced convulsions indicate that it does possess some convulsant effect. These results suggested possibility of involvement of GABAergic system and a direct excitatory action at level of neuromuscular junction by imidacloprid.

Key words: Experimentally induced convulsions, Imidacloprid, Toxicodynamic interactions.

INTRODUCTION

The biocidal agricultural chemicals, collectively known as pesticides, are the largest group of substances that are widely used in modern agricultural practices. Insecticides are also used in animal husbandry practices to control ticks, mites, fleas etc. So far many pesticides belonging to different chemical groups and having varied efficacy have been developed and proved very useful but their harmful effects cannot be ignored. Accidental, suicidal and homicidal poisonings leading to loss of human and animal life world over are common. Moreover, incidences of pesticide intoxications and death have been reported to be 13 times higher in developing countries.

Neonicotinoids represent the fastest-growing class of insecticides introduced to the market since the commercialization of pyrethroids (Nauen and Bretschneider, 2002). Like the naturally occurring nicotine, all neonicotinoids act on the insect central nervous system (CNS) as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Zhang *et al.,* 2000; Nauen *et al.,* 2001). Imidacloprid is a neonicotinoid insecticide and classified under toxicity class II /III agents by United States Environmental Protection Agency (USEPA, 1994). Imidacloprid and its analogs are remarkably potent neurotoxic insecticides, which act as nicotinic acetylcholine receptor agonists (nAChRs) (Matsuda *et al*., 2005). Although some studies have found low toxicity to mammals (Antra-Cordone and Durkin, 2005) and humans (David *et al.*, 2007).Moreover, immunological, biochemical and neurobehavioral deficits were found in rats exposed to imidacloprid (Abou-Donia *et al*., 2008; Bhardwaj *et al.,* 2010; Duzguner and Erdogan, 2010; Mohany *et al.,* 2011.) There are detailed studies of developmental immunotoxicity of imidacloprid in Wistar rats (Gawade *et al.,* 2013). But the literature about its mechanism of action in mammals is scanty. The present investigation was therefore undertaken to study the toxicodynamic interactions of imidacloprid on various neuropsychobehavioural parameters in mice/rats for better understanding of toxic mechanisms, developing remedial measures against toxicity and assessing safety evaluation for wide spread use of imidacloprid.

MATERIALS AND METHODS

Experimental animals

Swiss albino male mice weighing 20-25g and Wistar albino male rats weighing 100-120g were procured from Disease Free Small Animal Houseof the University and maintained in the Departmental Small Animal House. The animals were housed in polyacrylic cages kept at room temperature with a natural light-dark cycle. The animals were acclimatized to laboratory conditions for 3-4 days before the experiments were conducted. The animals were provided feed and water *ad libitum* and feed but not water was withdrawn 12h before and during experiments unless it was otherwise specifically stated for a particular parameter. All experiments were conducted between 9:00 a.m. and 5:00 p.m. in noise free laboratory conditions. The prior approval of Institutional Animal Ethics Committee for the protocol of this study was obtained and formulation of imidacloprid (Confidor, 200 SL) was used for this study.

For each experiment, mice (rats in lithium-pilocarpine induced status epilepticus) were randomly divided in three groups of 5 animals each. The control group was administered 10ml/kg of gum acacia solution (2%) and treatment groups received 22mg/kg or 44mg/kg ($2/5^{th}$ or $4/5^{th}$ of MTD) of imidacloprid intraperitoneally.

Assessment of effect of imidacloprid on experimentally induced convulsions

Supramaximal electroshock induced convulsions

The maximal electroshock seizure pattern was induced by giving electric shock (42 mA, 0.2 sec) using Electroconvulsiometer (Techno, India) through pinnal

electrodes, as described by Swinyard *et al*. (1952). Mice were screened 24h before the treatment and only those mice which showed tonic extension with electroshock, were selected for study. Observations were made ½h after treatment. Onset time of tonic flexion, tonic extension, clonic convulsions and duration (sec) of tonic extension and post tetanic depression and death, if any, were recorded. The increase in duration of tonic extension was considered as the index of epileptic activity and vice versa, as described by Toman and Guy (1964).

Pentylenetetrazole (PTZ) induced convulsions

Chemoshock/seizure pattern was induced by administering pentylenetetrazoleat the dose rate of 80 mg/kg subcutaneously as described by Swinyard *et al.* (1952). After ½h of treatment mice were administered pentylenetetrazole. Onset time of myoclonic jerks, clonic convulsions, tonic convulsions and death, if any, were noted.

Strychnine induced convulsions

The chemoshock seizures were induced by giving strychnine at the dose rate of 2.5 mg/kg subcutaneously. After 30 minutes of treatment, strychnine was administered to all mice. The onset, duration, nature and severity of convulsions and mortality, if any, were noted. The severity of convulsions was scored as described by Kulkarni and Jog (1983).

Piloerection, hyper locomotion	:	1
Stunning with catatonia	:	2
Clonic body tremors	:	3
Fore limb tonic flexor, extensor followed by clonic convulsions	:	4
Tonic extensor of both fore and hind limbs followed by clonus and death	:	5

Tremorine induced convulsions

Convulsions were induced using tremorine at the dose rate of 20 mg/kg body weight intraperitoneally. Treatment of mice in all the groups was done 30 minutes before challenging the mice with tremorine. Time taken for onset of tremors in limbs, duration of tremors and recovery and death, if any, were noted.

Picrotoxin induced convulsions

To study the effect onpicrotoxin induced convulsions, treatment of mice in all groups was done thirty min before picrotoxin administration through subcutaneous route at a dose rate of 3.5 mg/kg body weight. The onset, duration, nature, severity of convulsions and mortality, if any, were noted for each group. The severity of convulsions was scored as described by Kulkarni and Jog (1983).

Piloerection, hyper locomotion	:	1
Stunning with catatonia	:	2
Clonic body tremors	:	3
Fore limb tonic flexor, extensor followed by clonic convulsions	:	4
Tonic extensor of both fore and hind limbs followed by clonus and death	:	5

Lithium-pilocarpine induced status epilepticus

Effect of imidacloprid on status epilepticus induced by lithium– pilocarpine was studied using albino rats. Status epilepticus was induced by pilocarpine (30 mg/kg, i.p.) 24h after lithium sulphate (3 mEq/kg, i.p.).The effect of imidacloprid (40 mg/kg and 80 mg /kg i.e. 1/5th and 2/5th of LD_{50}) was studied on status epilepticus by administration of imidacloprid thirty min before administering pilocarpine. Control group received 10ml/kg of gum acacia solution (2%) intraperitoneally. The severity of status epilepticus was noted at 5, 15, 30, 45, 60, 90, 120, 180 and 240 min after treatment using scoring system as described by Patel *et al.* (1988):

No response stage	:	0
Fictive scratching stage	:	1
Tremors stage	:	2
Head nodding	:	3
Forelimb clonus stage	:	4
Rearing and falling back stage	:	5

Phenytoin-pentylenetetrazole inducedstatus epilepticus

Status epilepticus was induced in mice by the method of Rains *et al.* (1990). Phenytoin (50 mg/kg) was administered intraperitoneally in a volume of 10 ml/kg body weight to prevent the terminal tonic hind limb extension produced by pentylenetetrazole. Pentylenetetrazolewas administered 2h later, in a dose of 100 mg/kg, subcutaneously. The injection was made in the loose skin behind the neck, in a volume of 0.01 ml/10g body weight. Animals weretreated ½h before treatment with pentylenetetrazole. Time of onset of seizures and type and duration of seizures and mortality, if any, were noted. Seizure free state for a period of one hour was taken as protection.

Statistical analysis

Data was analysed by one way ANOVA followed by Dunnett's multiple comparison test. P values<0.05 were considered statistically significant.

RESULTS

Assessment of effect on supramaximal electroshock induced convulsions

Imidacloprid decreased the latency of tonic extension and significantly increased duration of tonic extension at both the dose levelsand delayed the onset of clonic convulsions (Table 1). The duration of post tetanic depression increased significantly at lower dose whereas it decreased significantly at higher dose.

Assessment of effect on Pentylenetetrazole induced convulsions

Imidacloprid increased the latency of myoclonic jerks and clonic convulsions nonsignificantly (Table 2). It

Table 1:
Effect of imidacloprid on supramaximal electroshock (42 mA, 0.2 sec) induced convulsions in mice*

Dose (mg/kg, i.p.)	Onset time (sec)			Duration (sec)		% mortality
	Tonic flexion	Tonic extension	Clonic convulsions	Tonic extension	Post tetanic depression	
Control	1.0	3.2±0.73	14.6±1.80	11.4±2.11^{A}	116.6±42.70^{A}	0
22	1.0	1.8±0.20	21.6±1.02	19.8±1.06^{B}	204.0±30.80^{B}	20
44	1.0	2.2±0.37	22.0±0.00	19.0±1.41^{B}	17.0±4.37^{C}	100

Table 2:
Effect of imidacloprid on pentylenetetrazole (80 mg/kg, s.c.) induced convulsions in mice*

Dose (mg/kg, i.p.)	Onset time (sec) after treatment with metrazol			% incidence of tonic convulsions	% mortality
	Myoclonic jerks	Clonic convulsions	Tonic convulsions		
Control	120.0±18.97	132.0±29.39	0.0±0.00	0	0
22	288.0±104.61	300.0±98.59	1020.0±120.00^{A}	60	60
44	216.0±77.30	180.0±65.72	420.0±129.61^{B}	80	80

*Values are mean ± SEM of five observations.
*Means bearing different superscripts differ significantly (P<0.05).

Table 3: Effect of imidacloprid on strychnine (2.5 mg/kg, s.c.) induced convulsions in mice*

Dose (mg/kg, i.p.)	Time of onset of convulsions (min)	Duration of convulsions (min)	Score (severity of symptoms)	% mortality
Control	8.2±1.65^{A}	1.5±0.31^{A}	5.0±0.00	100
22	5.8±2.03^{A}	1.5±0.44^{A}	4.8±0.20	100
44	2.7±0.85^{B}	4.7±1.25^{B}	4.2±0.47	100

Table 4:
Effect of imidacloprid on tremorine (20 mg/kg, i.p.) induced convulsions in mice*

Dose (mg/kg, i.p.)	Onset time of tremors (min)	Duration of tremors (min)	% mortality
Control	6.6±0.60	186.0±6.00^{A}	0
22	6.6±1.91	810.2±280.17^{B}	80
44	3.0±1.09	92.6±81.99^{A}	80

Table 5:
Effect of imidacloprid on picrotoxin (3.5 mg/kg, s.c.) induced convulsions in mice*

Dose (mg/kg, i.p.)	Onset of convulsions (min)	Duration of convulsions (min)	Score (severity of symptoms)	% mortality
Control	19.6±1.43^{A}	21.6±1.50^{A}	4.0±0.31	0
22	23.4±5.99^{A}	30.8±6.88^{A}	3.8±0.20	0
44	2.4±0.67^{B}	77.6±17.90^{B}	4.0±0.00	20

*Values are mean ± SEM of five observations. *Means bearing different superscripts differ significantly (P<0.05).

Table 6:
Effect of imidacloprid on lithium (lithium sulphate 3 mEq/kg, i.p.) followed by pilocarpine after 24 h (pilocarpine nitrate 30 mg/kg, i.p.) induced status epilepticus in rats

Dose (mg/kg, i.p.)	Score (severity of symptoms) at different time (min) after administration of pilocarpine								
	5	15	30	45	60	90	120	180	240
Control	0.0±0.00^{A}	2.0±0.94^{A}	4.6±0.40	4.6±0.40^{A}	4.0±0.63	3.0±0.54^{AB}	3.2±0.48^{A}	3.4±0.40^{A}	3.0±0.00
40	0.4±0.40^{A}	5.0±0.00^{B}	3.2±0.73	2.0±0.00^{B}	3.2±0.73	3.4±0.40^{A}	3.0±0.00^{A}	3.4±0.40^{A}	2.4±0.60
80	2.0±0.00^{B}	2.0±0.00^{A}	3.2±0.73	2.6±0.60^{B}	2.8±0.58	2.0±0.00^{B}	2.0±0.00^{B}	2.0±0.00^{B}	1.8±0.73

Table 7:
Effect of imidacloprid on phenytoin (50 mg/kg, i.p.) followed by pentylenetetrazole after 2h (100 mg/kg, s.c.) induced status epilepticus in mice*

Dose (mg/kg, i.p.)	Time of onset of convulsions (min)	Type of seizures	Duration of seizures (min)	% mortality
Control	3.2±0.66	Clonic	10.4±0.67^{A}	80
22	3.4±0.24	Clonic	9.6±1.40^{A}	100
44	3.0±1.08	Tonic, Clonic	35.0±0.50^{B}	100

Values are mean ± SEM of five observations. Means bearing different superscripts differ significantly (P<0.05).

also induced tonic convulsions in 60% and 80% of mice at lower and higher dose respectively which were absent in control group.

Assessment of effect on Strychnine induced convulsions Imidacloprid produced a significant decrease in onset time and increase in duration of convulsions at higher dose

only (Table 3). Mean seizures score was reduced though nonsignificantly in treated groups as compared to control.

Assessment of effect on Tremorine induced convulsions

Higher dose of imidacloprid decreased the latency of tremors and duration of tremors though nonsignificantly. Lower dose significantly increased the duration of tremors without affecting onset of tremors (Table 4).

Assessment of effect on Picrotoxin induced convulsions

Higher dose of imidacloprid decreased the latency but increased duration of convulsions significantly. Lower dose of imidacloprid increased the latency and duration of convulsions though nonsignificantly(Table 5).

Assessment of effect on Lithium-pilocarpine induced status epilepticus

Imidacloprid decreased latency as well as severity of status epilepticus in a dose dependent manner (Table 6) with the exception at 15 min when severity was maximum at lower dose.

Assessment of effect on Phenytoin-pentylenetetrazole induced status epilepticus

All the animals in control group showed unequivocal sustained clonic seizure activity. No effect on onset of convulsions was observed at both the dose levels. However the nature of convulsions was affected at higher dose i.e. spurts of tonic convulsions were also observed along with clonic convulsions. Duration of seizures significantly increased at higher dose level as compared to control (Table 7).

DISCUSSION

Seizure is characteristic feature associated with disordered and rhythmic high frequency discharge of impulse by a group of neurons in brain and status epilepticus is characterized by repeated episodes of epilepsy without the patient having recovered from previous attack.

The various models of seizures used in this study were maximal electroshock seizures (MES), pentylenetetrazole, strychnine, tremorine, picrotoxin, lithium-pilocarpine, phenytoin- pentylenetetrazole. Imidacloprid potentiated MES, however when given alone it produced CNS depression as well as tremors as indicated by gross observable behaviour profiles. Efficacy in MES test has been shown to correlate with the ability of drug to prevent partial and generalized tonic-clonic convulsions and cortical focal seizures in man and it is often stated that this model evaluates the capacity of drug to prevent seizure spread (James *et al*., 1964). Imidacloprid enhanced the severity of convulsions induced by pentylenetetrazole to tonic convulsions, and all animals showing tonic convulsions died. The pentylenetetrazoletest evaluates the ability of potential antiepileptic drugs to prevent clonic seizures and correlates with activity against these seizures (Desai *et al*., 1995). The underlined neuronal abnormality is poorly understood.

In strychnine induced convulsions, imidacloprid induced earlier onset of convulsions with increased duration at higher dose only. So imidacloprid at higher dose level interferes with the action of strychnine which is known to be mediated by inhibition of release of glycine. Also imidacloprid potentiated the effect of tremorine. In picrotoxin induced convulsions, higher dose of imidacloprid decreased the latency of convulsions. Picrotoxin is reported to act through the GABA receptor complex and Cl^- channel. The potentiating effect of imidacloprid on picrotoxin action indicates involvement of GABAergic system and Cl^- channel. In lithium-pilocarpine treated animals imidacloprid decreased severity of status epilepticus. Lithium pre-treatment provokes limbic seizures following administration of sub-convulsive dose of pilocarpine. The combined treatment caused accumulation of ACh and inositol monophosphate and reduced cortical inositol. Increased severity of seizures seen initially might be due to potentiation of limbic seizures. In case of phenytoin-PTZ induced status epilepticus higher dose of imidacloprid induced tonic convulsions. This might be due to early activation of Na^+ channels or potentiation of effect of PTZ. Imidacloprid potentiated the experimentally induced convulsions to the highest degree i.e. tonic convulsions or death in all the models except status epilepticus induced by lithium pilocarpine. In lithium-pilocarpine induced status epilepticus the severity of convulsions was reduced. Antiepileptic drugs are CNS depressant and anxiogenic. Imidacloprid also produces depressant symptoms as well as tremors. Hypothetically it can be said that mechanism of action of imidacloprid responsible for tremors enhances the action of epileptic agents. The effect of imidacloprid on experimentally induced convulsions indicates that it does possess some convulsant effect. This may be due to its nicotinic cholinergic receptor stimulation leading to initiation in end plate potential in muscles or an excitatory post synaptic potential in nerve.

Some protection from experimentally induced convulsions by other group of insecticides in mice has been observed thus showing some anticonvulsant activity (Malik, 1975; Thaker *et al*., 1985; Garg *et al*., 1987; Punia *et al*., 1995).

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Research Article

STUDIES ON HEMATOLOGICAL AND HISTOPATHOLOGICAL CHANGES FOLLOWING SUBCHRONIC EXPOSURE OF ACETAMIPRID IN MICE

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ABSTRACT

The present study was carried out to assess the toxicological effect of acetamiprid, a new insecticide of neonicotinoid group on hematological and histopathological parameters in adult male Swiss albino mice. The experimental animals were divided into three different groups of six animals each. The first group was control group given 3% gum acacia while other two treatment groups were administered acetamiprid 2.3 and 4.6 mg/kg/day i.p. to mice daily for 60 and 90 days. There was significant decrease in Hb value observed in 90 days treated group only. Significant differences were observed in MCV, MCH and MCHC in 90 days treated group and also in MCHC in 60 days treated group. TLC decreased significantly with the increase in treatment level in 60 and 90 days treatment group. Various histopathological lesions were observed in liver, kidney and testis. Central lobular necrosis with congestion of central vein, formation of new bile duct, diffused hemorrhages, infiltration of mononuclear cells around the central vein were observed in the liver. In kidney, tubular necrosis surrounded by infiltration of mononuclear cells, hydropic degeneration and presence of various casts and hemorrhages and atrophy of glomerulus were observed. In testes, there was detachment and degeneration of spermatogonia from seminiferous tubules. Reduced concentration of sperms in seminiferous tubules was also observed.

Key words: Acetamiprid, subchronic, hematology, histopathology.

INTRODUCTION

At present, use of pesticides in agricultural production is increasing considerably throughout the world due to the rise in population and crop production. As much as 4.6 million tons of pesticides are released annually into the environment and approx. 500 types of these pesticides are considered a threat to the environment and human beings (Zhang *et al.*, 2011). Worldwide annual sale of neonicotinoids is approximately USD one billion, accounting for 11-15% of the total insecticide market (Tomizawa and Casida , 2005).

Acetamiprid, a new member of neonicotinoid synthetic chlorinated insecticide family has been recently introduced in the market and is highly effective for controlling aphids, beetles, moth, leafhopper, pests on crops and leafy vegetables, along with fleas infesting livestock and pet animals. It is a systemic insecticide with translaminar action which has a contact and stomach action (Zhang *et al.*, 2010). Acetamiprid is used against insects that have gained resistance to organophosphate, carbamate and synthetic pyrethroid (Si *et al.*, 2005).

Although acetamiprid is widely used in India and abroad, but there is paucity of data on its toxicity in animals. Therefore, the present study was carried out to evaluate the influence of subchronic acetamiprid exposure on haematological and histopathological changes in male mice.

MATERIALS AND METHODS

Experimental animal

Adult Swiss albino male mice weighing between 17-27 g were procured from Disease Free Small Animal House of the Institute and acclimatized in laboratory conditions for a period of 7 days prior to experimentation. In the Departmental Animal House, the animals were housed in polyacrylic cages in group of six per cage at room temperature with a natural light-dark cycle and provided feed and water *ad libitum*. Animal house temperature varied between 22 to 27°C throughout the experiment. The prior approval of Institutional Animal Ethics Committee was obtained for use of the laboratory animals in this study and the experiments were conducted as per guidelines of CPCSEA.

Chemicals

Technical grade Acetamiprid (ACE) (96.8% pure) was procured from Tropical Agrosystem (India) Pvt. Limited, Chennai.

Experimental design

Adult male Swiss albino mice were divided into three groups comprising of six animals each. Group I (vehicle control) animals were administered 3% aqueous solution of gum acacia and Group II and III (treatments) were administered acetamiprid 2.3 and 4.6 mg/kg body weight in 3% aqueous solution of gum acacia (1/20 and 1/10 of MTD), respectively. Vehicle and acetamiprid were administered to mice daily by i.p. route for 60 and 90 days. The desired concentration of acetamiprid was prepared in 3% aqueous solution of gum acacia, as vehicle. Body weight of animals was recorded regularly. At the end of experiment, mice were sacrificed using mild ether anesthesia. Blood from individual animal was collected from heart at the time of sacrifice using dry sterilized vials containing anticoagulant, sodium EDTA. Various

hematological parameters viz. hemoglobin concentration (Hb), packed cell volume (PCV), total erythrocytes count (TEC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using automated hematology analyzer.

Patho-morphological examinations

At the end of experiment, mice were sacrificed. Macroscopic lesions in liver, kidney and testis were recorded and representative pieces of tissues from these organs were collected in 10% formal saline solution. Preserved samples were processed in ascending grade of alcohol and cleared in xylene, embedded in paraffin, sectioned at 4-5 µm thickness and stained with haematoxylin and eosin (H&E) 16 (Luna, 1968).

Statistical analysis

The data was expressed as Mean ± SEM and analyzed by using SPSS statistics 17 software. The data was compared by employing one way ANOVA with Duncan's multiple comparisons as post hoc test. A p-value of <0.05 was considered as statistically significant.

RESULTS

Effect of acetamiprid on body weight

The results show non-significant increase in body weight in each group (Table 1).The results show that there is decrease in value of Hb in treated groups as compared to control group, but significant difference was observed in 90 days treated group only. Other hematological parameters were also decreased with increase in treatment level except MCHC which was increased. Significant differences were observed in MCV, MCH and MCHC in 90 days treated group and also in MCHC in 60 days treated group. TLC was decreased with increase in treatment level and significant difference was observed in 60 and 90 days treatment group (Table 2).

The histopathological examination of control mouse liver tissue revealed radially arranged hepatic cords around the central vein, while the liver of the mice treated with acetamiprid showed central lobular necrosis with congestion of central vein, formation of new bile duct, diffused hemorrhages, infiltration of mononuclear cells around the central vein (Fig. 1). Kidney of acetamiprid treated mice showed tubular necrosis surrounded by infiltration of mononuclear cells, hydropic degeneration and presence of various casts and hemorrhages and atrophy of glomerulus (Fig. 2 and 3). Testis of acetamiprid treated mice revealed detachment and degeneration of spermatogonial from seminiferous tubules, reduced concentration of sperms in seminiferous tubules, edematous changes and congestion of blood vessels in interstitial space (Fig.4).

Table 1:
Effect of subchronic toxicity study of acetamiprid on body weights of mice

Treatment (mg/kg, i.p.)	Mean body weight in grams (mean ± SE) Days post acetamiprid administration						
	60 Days			90 Days			
	0	30	60	0	30	60	90
Control(300)	15.66^{a}±0.92	27.00^{a}±0.93	32.33^{a}±0.76	14.00^{a}±0.73	26.00^{a}±1.39	32.50^{a}±0.81	34.00^{a}±0.58
ACE(2.3)	14.83^{a}±0.31	25.17^{a}±0.31	29.83^{a}±0.65	14.67^{a}±0.49	26.17^{a}±0.75	29.67^{a}±1.14	33.33^{a}±1.14
ACE(4.6)	15.17^{a}±0.31	27.17^{a}±0.60	30.00^{a}±1.00	16.33^{a}±0.99	28.33^{a}±1.35	31.50^{a}±0.84	34.67^{a}±0.95

Values are mean ± SE; n=6; ACE: Acetamiprid; Values bearing common superscripts within column of a parameter do not differ significantly at 5% level of significance

Table 2:
Effect of toxicity of acetamiprid on various haematological parameters in mice

Expt.	Treatment (mg/kg, i.p.)	Hb (g/dl)	TEC (x10^{6} /cmm)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	TLC (x10^{3} /cmm)	DLC (no. of cells/100 leukocyte) N	L	M	E	B
	Control	14.60a	9.98a	47.42a	52.63a	15.63a	28.88a	10.68b	24.41	70.93	2.45	0.43	1.76
	(300)	±0.58	±0.42	±0.61	±1.49	±0.39	±0.27	±1.32	±4.36	±3.36	±1.36	±011	±0.42
60	ACE	14.37a	9.68a	47.02a	50.13a	14.92a	29.70ab	8.48ab	33.36	63.86	1.3	0.42	1.06
days	(2.3)	±0.33	±0.27	±1.44	±0.48	±0.26	±0.28	±0.88	±4.26	±4.26	±0.51	±0.11	±0.25
	ACE	13.88a	9.18a	45.88a	50.02a	15.12a	30.26b	7.30a	26.75	71.8	0.9	0.35	0.9
	(4.6)	±0.19	±0.19	±0.90	±0.57	±0.24	±0.31	±2.44	±5.75	±6.70	±0.00	±0.25	±0.00
	Control	15.10a	9.98a	51.68a	52.63a	15.63a	27.70a	13.02a	24.41	70.93	2.45	0.43	1.76
	(300)	±0.26	±0.42	±1.53	±1.49	±0.39	±0.52	±0.58	±4.36	±3.36	±1.36	±0.11	±0.42
90	ACE	14.67ab	9.72a	50.91a	52.35a	15.06ab	28.80ab	12.59a	28.1	69	1.2	0.15	1.55
days	(2.3)	±0.19	±0.64	±0.87	±0.57	±0.12	±0.20	±0.63	±6.10	±4.90	±0.80	±0.05	±0.45
	ACE	13.93b	9.41a	43.23a	48.23b	14.28b	29.60b	8.76b	27.8	69.95	1.6	0.3	0.35
	(4.6)	±0.44	±0.43	±435	±0.29	±0.22	±0.34	±1.95	±9.20	±9.55	±0.30	±0.30	±0.09

Values are Mean ± SE; n=6; Values bearing common superscripts within column of a parameter do not differ significantly at 5% level of significance; ACE: Acetamiprid

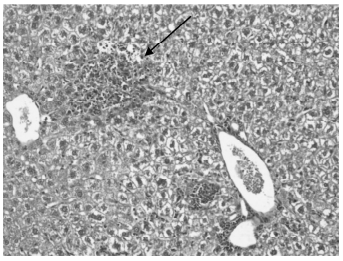
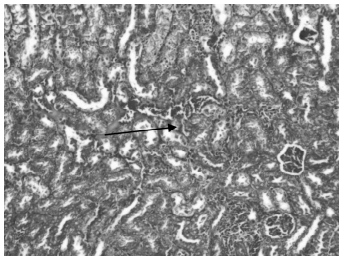


Fig. 1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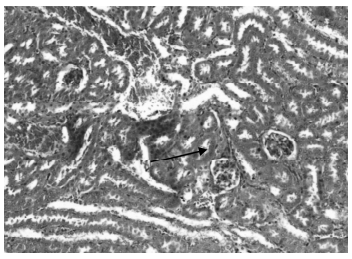
Fig. 3

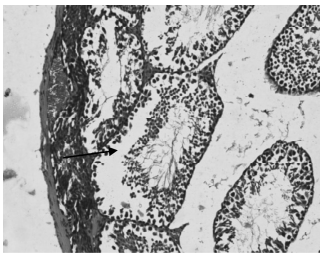
Fig. 4

DISCUSSION

The results of the present study revealed that i.p. administration of acetamiprid for a period of 90 days exhibited significant decrease in Hb of acetamiprid treated male Swiss albino mice. Reduction in hemoglobin content could be due to impaired biosynthesis of heme in bone marrow (Shakoori *et al.*, 1992). Further, the reduction in Hb could also be due to increased rate of destruction or reduction in the rate of formation of RBCs. This premise is supported by low TEC values as observed in acetamiprid treated mice. In an experiment conducted with organochlorine insecticide lindane it was indicated that lindane induced hemolysis, hemorrhage and/ or reduced erythropoiesis in various species of birds and this could account for the observed decline of TEC population (Mandal *et al.*, 1986).The reduction in blood parameters (PCV, TEC, Hb) may be attributed to a hyperactivity of bone marrow (Tung *et al.*, 1975) leading to production of red blood cells with impaired integrity which were easily destructed in the circulation. It has been suggested that decrease in RBC count due to talastar toxicity is either indicative of excessive damage to erythrocytes or inhibition of erythrocyte formation in rabbits (Shakoori *et al.*, 1990). The decrease in PCV is obviously contributed by the decreased cellular count in blood after pesticide treatment. Similar effects have also been found in sheep (Yousef *et al.*, 1998) due to cypermethrin and dimethoate toxicity and in male Wistar rats due to acetamiprid oral exposure of 28 days (Doltade *et al.*, 2012). Alterations in PCV, MCV and MCHC indicated that acetamiprid caused adverse effect on hematopoietic system of mice after subchronic exposure. There was significant decrease in TLC. Similarly a significant decrease in total leukocyte count was observed in female rats after oral administration of acetamiprid for 28 days (Mondal *et al.*, 2009) whereas non significant decrease in total leukocyte count was observed in male mice after i.p. administration of acetamiprid for 30 days (Preeti *et al.*, 2014). Results of present study revealed that exposure to acetamiprid resulted in histological alterations in liver, kidney and testis of mice. Liver sections displayed moderate degenerative changes *viz.* single cell necrosis in centrilobular region and single cell necrosis of hepatocytes and focal area of hemorrhage. Similar observations have been reported after repeated exposure of high dose of another neonicotinoid, imidacloprid (20 mg/kg/d) for 90 days in female rats wherein the hepatocytes of liver showed mild focal necrosis with swollen cellular nuclei and cytoplasmic lesions (Bhardwaj *et al.*, 2010). Kidney of acetamiprid treated mice showed cellular swelling of distal convoluted tubules, mild degeneration of proximal convoluted tubules and glomerular atrophy. Histopathological alterations in kidney indicated that acetamiprid produces nephrotoxicity in addition to hepatotoxicity. Testis of acetamiprid treated mice revealed detachment and degeneration of spermatogonial from seminiferous tubules, reduced concentration of sperms in seminiferous tubules, edematous changes and congestion of blood vessels in interstitial space. Similar findings in albino mice have also been reported earlier by Chawsheen (2011) and Preeti (2014).

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Research Article

MECHANISTIC DIFFERENTIATION OF THE MUSCARINIC RECEPTORS MEDIATING CONTRACTION IN ISOLATED RETICULAR GROOVE OF ADULT MALE GOATS

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ABSTRACT

The closure of reticular groove (RG) is important in adult ruminants for direct delivery of drugs, nutrients, etc. There is no information available on physiopharmacology of RG of goats. Hence, in the present study, effects of acetylcholine (ACh) and its antagonists were evaluated on isolated reticular groove strip (IRGS) preparation of adult male goats. The mechanical activity of IRGS was isometrically recorded under a resting tension of 1.5 g in a thermostatically controlled (37±1°C) organ bath (UGO Basile, Italy). The muscarinic receptor blockers atropine, pirenzepine and 4-DAMP evoked significant reduction of sub-maximal concentration-dependent contractile response of ACh whereas methoctramine had no antagonistic effect. Our results showed that M_3 receptor blocker exhibited inhibition of sub-maximal concentration-dependent contractile response of ACh at 10 fold lower concentration, as compared to M1 receptor blocker, thereby, suggesting that the ACh-induced tonicity of RG might be mainly mediated via stimulation of M_3 receptors.

Key Words: Acetylcholine, goat, muscarinic receptor, reticular groove, ruminant stomach

INTRODUCTION

In suckling ruminants, stomach is similar to the mono-gastric animals. The liquid food directly goes to the abomasum through functional reticular groove (RG), which is a muscular structure and has two well-defined lips or pillars at the lower end of the esophagus and runs along the medial internal wall of the reticulum and omasum then terminates at glandular compartment of abomasum.

Reflex contraction of the RG has been observed in young ruminants and a reflex arc has been described by section and stimulation of afferent and efferent nerves (Comline and Titchen, 1951). The RG receives a pre-ganglionic para-sympathetic innervation (abdominal vagal trunk). The gastrointestinal motility in mammalian species including cattle, is driven by the autonomic nervous system (Bornstein *et al*., 2004). The motility can be generated and modulated by local and circulating neurohumoral substances (Hansen, 2003). It has been suggested that the reflex closure of RG may be under central and local control (Reid and Titchen, 1988). The intrinsic contractions have also been recorded *in vitro* from strips of reticular and ruminal muscles of sheep, and described the pattern of contractions of the ruminal strips that consist a rhythmic series of contractions (Dussardier and Navarro, 1953). The administration of atropine exerts an inhibitory effect on the constriction of the RG in calves which was accompanied atony of the reticulorumen (Braun *et al*., 2002). The bovine RG has numerous autonomic ganglia, mostly composed of acetylcholinesterase (AChE)-positive neurons. Barahona *et al*. (1997) suggested a wide distributed AChE-positive nerve fibers through muscle layers of the RG in cattle by AChE-immunohistochemistry.

Goat is very important ruminant in Indian subcontinent. During our efforts to improve effects of some anthelmintic in goats, we administered prescribed salt solutions such as, $ZnSO_4$, $CuSO_4$, $NaHCO_3$ and $CoSO_4$, prior to administration of anthelmintic, so as to close the RG. But to our utter surprise it did not work. Nevertheless, closure of RG in ruminants has relevant implications in the utilization of protein supplement, oral glucose therapy in ketosis, etc. and absorption and efficacy of therapeutic agents. But the role of parasympathetic nervous system and post-junctional muscarinic receptor subtypes involving in activity of the RG smooth muscle of goat is not known and to the best of our knowledge, there is no information available on the neuropharmacology particularly with regard to the functioning of cholinergic system in the RG of adult goats, hence, the present study was conducted to evaluate effect of ACh and to functionally differentiate its receptor subtypes in RG of adult goats.

MATERIALS AND METHODS

Preparation of isolated reticular groove strips (IRGS)

Reticular groove of freshly slaughtered adult male goat was collected in Tyrode's solution (4°C) from local abattoir and brought to the laboratory. The tissue was placed in a Petri dish containing Tyrode's solution. The smooth muscle was freed from the mucosa and submucosa. Strips of transverse muscle (approximately 2-3 mm wide and 10-12 mm long) were cut parallel to the orientation of the muscle fibers, prepared from the floor of RG. Strips were mounted between two hooks made from 37 gauge stainless steel wire and kept under a resting tension of 1.5 g (the optimal passive tension was

determined by tension-stretch experiment using 80 mM KCl solution) and allowed to equilibrate for 60 min in a thermostatically controlled (37±1°C) organ bath (UGO Basile, Italy) of 10 ml capacity containing Tyrode's solution and continuously bubbled with medical gas (74% N_2 + 21% O_2 + 5% CO_2). The viability of tissue was tested by stimulating the preparations with 80 mM KCl. Concentration-response curves were produced by exposing strips to 10^{-8}-10^{-3} M ACh in a non-cumulative manner, each wash was given when the response to the concentration reached a maximum, then incremental concentrations were added. To determine the effect of the muscarinic antagonists (atropine, pirenzepine, methoctramine and 4-DAMP), the preparations were incubated with the antagonist for 30 min after a sub-maximal concentration-response to ACh was constructed, then sub-maximal concentration of ACh was added.

Drugs and solutions

The composition of Tyrode's solution: NaCl 137, KCl 2.7, $CaCl_2.2H_2O$ 1.8, $MgCl_2.6H_2O$ 1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, D-Glucose 5.5 mmol/L (Emendorfer *et al.,* 2005; Desire *et al.,* 2010). Distilled water was used to prepare stock solution.

The following drugs were procured from Sigma-Aldrich, USA: acetylcholine chloride, atropine sulphate, hexamethonium, pirenzepine dihydrochloride, methoctramine hydrateand, 4-diphenylacetoxy-N- methyl-pipiridinemethiodide(4-DAMP). 4-DAMP was dissolved in DMSO and other drugs were dissolved in distilled water.

Calculation and statistical analysis

The effects of the test compounds were expressed as absolute gram tension. The results are presented as the mean ± SEM with 'n' equal to number of animals. Both Emax (the maximal response) and EC_{50} (the concentration producing 50% of the maximal response) were determined by nonlinear regression analysis (sigmoidal dose response with variable slop) using GraphPad Prism version 4.00 (San Diego, California, USA). Sensitivity/potency was expressed as pD_2 = -log EC_{50}. Data were analyzed by unpaired 't' test. Differences in values were considered statistically significant at $P<$ 0.05.

RESULTS

Ach caused concentration-dependent (10^{-8}-10^{-3}M) contraction of the muscle strips from the floor of the RG (Fig.1). The contractile response to ACh was 0.54 ± 0.04 and 0.75 ± 0.03 g at sub-maximal (10^{-4} M) and maximal (10^{-3} M) concentrations, respectively. The pD2 value was calculated as 4.33. The excitatory effect of sub-maximal dose of ACh was attenuated by incubation of muscle strips with the non-specific muscarinic receptor antagonist atropine at a very low concentration i.e. 10^{-8} M. Atropine evoked a significant inhibition of sub-maximal concentration response of ACh (Fig 2, 5a).

We used specific muscarinic receptor antagonists, pirenzepine (M_1) and 4-DAMP (M_3) in a concentration of 1µM and 0.1 µM, respectively. Pirenzepine and 4-DAMP also evoked a significant inhibition of sub-maximal concentration response of ACh (Fig 3,4,5b,5c). However, methoctramine (M_2 receptor antagonist) had no antagonistic effect even at 0.1 mM concentration.

DISCUSSION

Analysis of results of the present work reported here shows presence ACh receptors on RG of adult goats

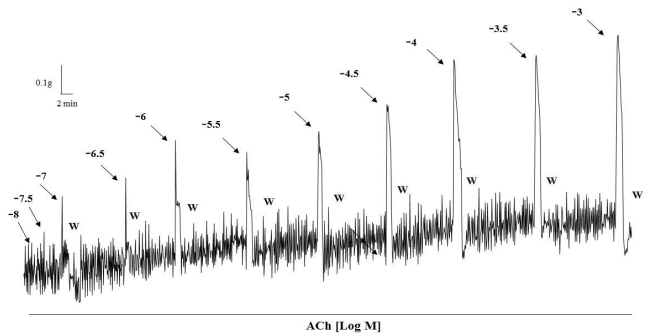

Fig. 1:
Representative tracing of concentration-dependent contractile response of isolated reticular groove strip of adult goat to non-cumulative doses of ACh (10^{-8}-10^{-3}M).

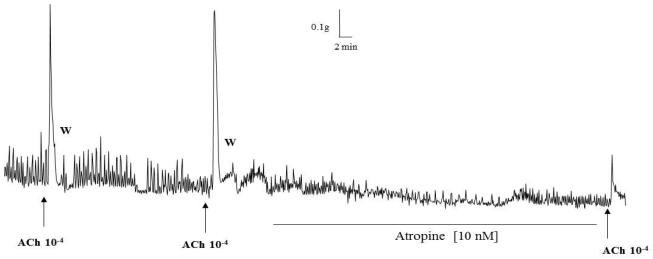

Fig. 2:
Representative tracing showing inhibitory effect of atropine [10 nM] on contractile response of isolated reticular groove strip of adult goat to sub-maximal dose of ACh (10^{-4} M).

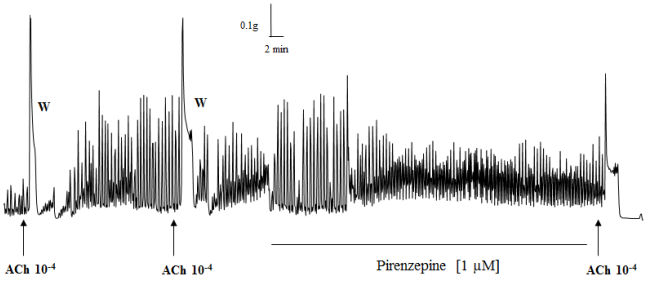

Fig. 3:
Representative tracing showing inhibitory effect of pirenzepine [1 µM] on contractile response of isolated reticular groove strip of adult goat to sub-maximal dose of ACh (10^{-4} M).

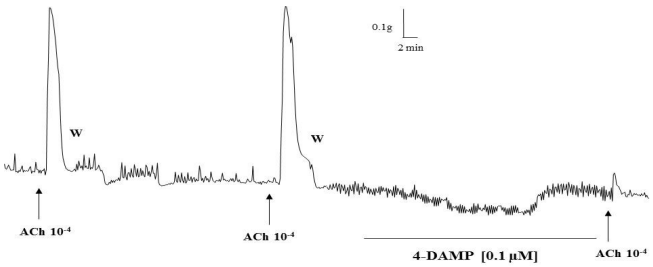

Fig. 4: Representative tracing showing inhibitory effect of 4-DAMP [0.1 µM] on contractile response of isolated reticular groove strip of adult goat to sub-maximal dose of ACh (10^{-4} M).

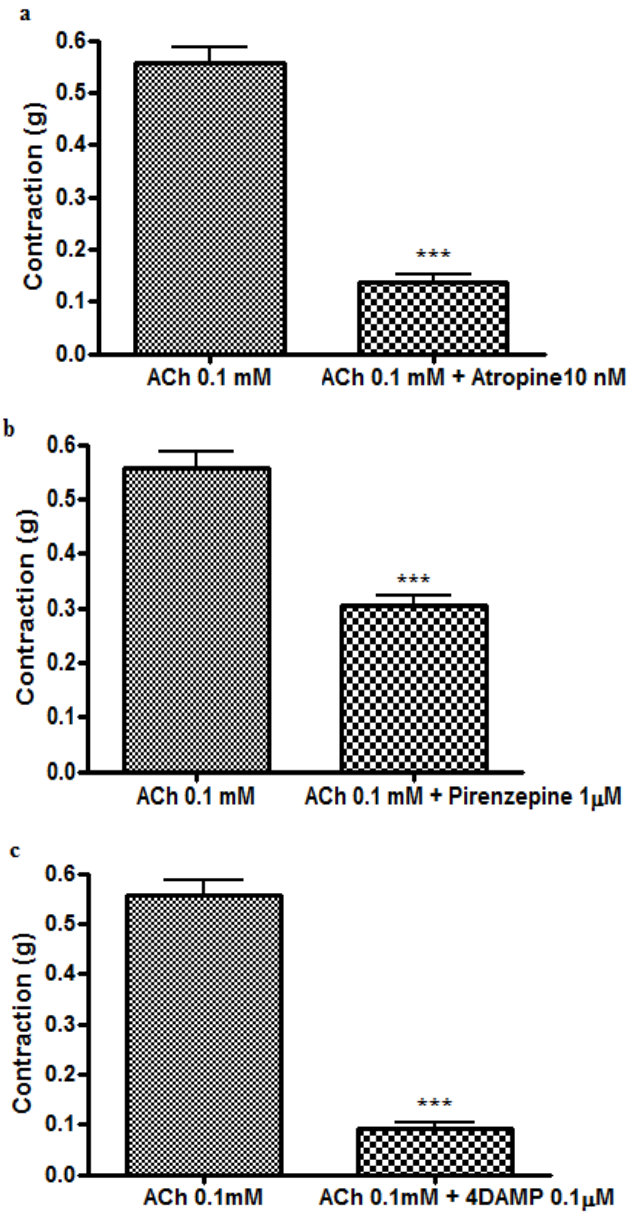

Fig. 5:
Graphical presentation showing significant inhibitory effects of atropine (10 nM) (a), pirenzepine (1 µM) (b) and 4DAMP (0.1 µM) (c).Vertical bars represent SEM *** P< 0.001 in comparison to control.

because ACh has *in vitro* induced concentration-dependent (non-cumulative) increase in basal tone. The findings are consistent with the effect of ACh like agonist bethanechol described for the stomach, duodenum, jejunum, cecum, and pelvic flexure of horses (Ringger *et al*., 1996; Marti *et al*., 2005) stomach and small intestines of rats (Megens *et al*., 1991), ileum, cecum, and right ventral colon of ponies (Lester *et al*., 1998). To determine the mAChR subtypes which are attributed the cholinergic contraction induced by ACh, study was designed that included pre-incubation with mAChR antagonists. The anticholinergic agent atropine (10^{-8} M) significantly abolished the effects of ACh confirmed that the effect of ACh is mediated via mAChRs. Pre-incubation with the ganglionic blocker hexamethonium (10^{-4} M) had no effect on the excitatory effect of ACh.

Although the selectivity of the ACh is very low, from our agonist-antagonists study the mediation of M_1 and M_3 receptors could be suggested in the contraction of the smooth muscle of the RG. The specific mAChR antagonists, depending on their affinity patterns used in the experiment were determined from previous reported studies i.e. M_1-receptor described in smooth muscle (Bloom *et al*., 1987), and it is most sensitive to the antagonist pirenzepine (Doods *et al*., 1987). The intermediate affinity of the M_1-muscarinic antagonist, pirenzepine, is reported for the M_3-receptor in visceral and vascular smooth muscle (Vockert *et al*., 1993; Pfaff *et al*., 1995). The pA_2 value of pirenzepine in bovine RG (Barahona *et al*., 1997) was similar to those found in the ileum (Kurtel *et al*., 1990) of the guinea-pig, longitudinal muscle of human colon (Kerr *et al*., 1995), rat duodenum (Pfaff *et al*., 1995) as well as in the bronchi and bronchiole of the dog (Itabashi *et al*., 1991). Thus, the affinity pattern for pirenzepine (Grimm *et al*., 1994) is M_1>M_3>M_2. The mAChRs M_1 have also been detected in the myenteric and submucosus plexus of the bovine gastro-intestinal tract by use of immunohistochemical technique (Stoffel *et al*., 2006). The pA_2 value was lower for methoctramine in bovine RG (Barahona, *et al*., 1997) to that observed for M_2 in guinea-pig atria (Eglen *et al*., 1988), and the affinity pattern for methoctramine (Grimm *et al*., 1994) is M_2>M_1>M_3. The present study demonstrate M_2 receptor antagonist, methoctramine could not affect the contractile effect at sub-maximal dose of ACh, suggested devoid of M_2 muscarinic receptors and not involved in mediation of the contractile effect of ACh. Earlier study in cattle (Barahona *et al*., 1997) suggested the competitive inhibitory effect of methoctramine confirmed the presence of M_2 muscarinic receptor. Several Studies indicate synergistic effects of co-localised M_2 and M_3 receptors in controlling smooth muscle contraction (Ehlert, 2003)

Apart from M_1-receptor, M_3-receptor had a major significant role in contractile effect of ACh in RG of adult

goat. Subtype M_3-receptor was described in glandular tissues and smooth muscle exhibited a high affinity for 4-DAMP (Eltze and Figala, 1988). The receptors activated by ACh could be of the M_3 subtype is supported by competitive inhibition shown by 4-DAMP. Barahona *et al*. (1997) suggested the pA_2 value of 4-DAMP in cattle is very close to that reported for M_3 receptor (Grider *et al*., 1987; Kurtel *et al*., 1990), and also similar to those found in the guinea-pig ileum (Choo *et al*., 1988), common bile duct (Karahan *et al*., 1991) of the guinea-pig and the circular muscle of human colon (Kerr *et al*., 1995). Due to the fact that 4-DAMP is a muscarinic antagonist that shows high affinity not only for the M_3 but also for the M_1 subtype receptors (Hulme *et al*., 1990). The antagonist patterns for 4-DAMP is M_3>M_1>M_2 (Eltze *et al*., 1993; Grimm *et al*., 1994). Furthermore, it has been demonstrated the bethanechol increased contractility predominantly through M_3 and, to a lesser extent, through M_2 in smooth muscle preparation of intestine (Buehler *et al*., 2008) and the abomasal antrum (Michel *et al*., 2003) of dairy cows.

Furthermore, analysis of the results of our study involving mAChR antagonists indicated that the effect of ACh was primarily mediated via M_3 mAChRs and, with a minor role, via M_1 mAChRs. A similar effect of cholinergic receptor agonists (ACh**,** bethanechol) have been reported for the RG (Barahona *et al*., 1997), abomasum, ileum, and large intestine of cattle *in vitro* (Michel *et al*., 2003). On the basis of functional *in vitro* study, we can conclude that the ACh-induced contraction is mediated via stimulation of M_3-muscarinic receptors in the goat RG smooth muscle. These findings may openup opportunities for improved understanding of the subtype specificity of mAChR modulation of RG function and for the development of new drugs selectively targeting different subtypes of mAChRs.

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Research Article

BILIRUBIN ACCELERATE CUTANEOUS WOUND HEALING IN EXCISION WOUND MODEL OF RATS

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ABSTRACT

Bilirubin, a by-product of heme degradation, has an important role in cellular protection and cutaneous wound healing potential in some preliminary studies. Here we hypothesize that bilirubin facilitates wound healing in rats in a time-dependent manner. Full thickness cutaneous wounds were created under pentobarbitone anaesthesia. All the rats were divided into two groups, of which one (control) was treated with ointment base and other with bilirubin ointment (0.3%). Wound closer measurement and tissue collection were done on days 3, 7, 11 and 14 post-wounding. Histopathological changes (cellular migration, fibroblast deposition, epithlialization, blood vessels formation, collagen deposition) were assessed by H&E staining. The percent wound closer was significantly higher on days 7, 11 and 14 in bilirubin-treated rats as compared to control. Bilirubin markedly facilitated cutaneous wound healing in rats by recruitment of inflammatory cells, deposition of fibroblast cells, formation of new blood vessels and epithelialization to the wound site. Therefore, topical application of bilirubin ointment might be of great use in cutaneous wound healing in rats.

Keywords Bilirubin; cutaneous wound healing; Percent contraction; H & E

INTRODUCTION

Wound healing is a complex and well-orchestrated process of replacing devitalized cellular structures and tissue layers that are produced after surgical procedures and traumatic injury. It involves four overlapping stages viz., heamostasis, inflammation, proliferation and remodeling. During the inflammation, significantly increased inflammatory cells (Singer and Clark, 1999) and produces sudden burst of reactive oxygen species (ROS) in the wound tissue. Although oxidants play an important role in wound healing providing signaling and defence against microorganisms. Overproduced ROS results into oxidative stress, with detrimental cytotoxic effects causing delayed wound healing (Clark, 2008). Hence, the oxidants have to be detoxified in order to prevent damage to host cells.

Bilirubin, a breakdown product of normal heme catabolism. The biological action of bilirubin is relevant to the prevention of oxidant-mediated cell death (Kushida *et al*., 2003). Bilirubin at a low concentration scavenges reactive oxygen species (ROS) *in vitro*, thereby, reducing oxidant-mediated cellular damage and attenuating oxidant stress *in vivo*. Bilirubin is a potential free radical scavenger (Thomas *et al*., 2009). The roles of biliverdin and bilirubin in counteracting oxidative and nitrosative stress have been reviewed extensively by Morse and Choi (2005). In view of the above, it was hypothesized that bilirubin might help in wound healing by attenuating oxidative stress.

MATERIALS AND METHODS

Animals used

Forty animals were used in this study, which were divided into two groups i.e., group I (control) and group II (treated) consisted of twenty animals in each group.

Creation of wound in rats

All animals were anaesthetized by intraperitoneal (i.p.) injection of pentobarbitone sodium (@ 50 mg/kg). Approximately 2 x 2 cm^2 (400 mm^2) open excision-type wound was created on the back (dorsal thoracic region) of the rats to the depth of loose subcutaneous tissue. After recovery from anaesthesia, animals were housed individually in properly disinfected cages.

Application of ointment

All rats were divided into two groups consisting of 20 animals and each group was further subdivided into 4 subgroups with 5 animals in each group.

1. Group I (control): Wound was topically treated with ointment base (5% hard paraffin, 90% soft paraffin and 5% lanolin) twice daily.
2. Group II (bilirubin-treated): Wound was topically treated with 0.3% bilirubin ointment, applied twice daily.

Wound contraction measurement

Wound area was measured on days 0, 3, 7, 11 and 14 post-wounding by tracing its contour using a transparent paper. The area (mm^2) within the boundaries of each tracing was determined planimetrically and expressed as per cent wound contraction. The values were expressed as percent values of the 0 day measurement and were calculated by the formula:

$$\text{\% wound contraction} = \frac{\text{0 day wound area-wound area on particular day}}{\text{0 day wound area}} \times 100$$

Collection of tissue for histopathological study by H&E staining

Five rats from both the groups were killed on days 3, 7, 11 and 14 to collect granulation tissue and preserved in 10% neutral buffered formalin for histopathological study. The fixed granulation tissues were embedded in paraffin. 5 µm thick tissue sections were stained with H&E as per standard procedure. The stained sections were visualized under light microscope (OLYMPUS, BX 41, USA) at 40x magnification. Ten random fields (40x) from different sections in each group were evaluated and scoring (1-15) was done according to standard method.

Statistical Analysis

Results are expressed as mean±S.E. and analyzed by applying two-way ANOVA using GraphPad Prism v4.03 software program (San Diego, CA, USA) and the differences between the experimental groups were considered statistically significant at $P<0.05$.

RESULTS

Fig. 1a shows the gross appearance of wound in bilirubin-treated and control rats on different days (0, 3, 7, 11, and 14) and it also depicts progressively greater wound closer in bilirubin-treated rats on day 7 onwards, as compared to control. The per cent wound closer was significantly greater on days 7, 14 and 19 in bilirubin-treated rats, as compared to respective day control rats (Fig. 1b).

Fig. 2 shows the representative pictures of H&E stained sections of granulation/healing tissue of bilirubin-treated and control rats on different days. On day 3, bilirubin-treated group showed more migration of inflammatory cells, as compared to control. The proliferation of fibroblasts, collagen deposition and formation of blood vessels were more evident in bilirubin-treated, as compared to control group. On day 7, the control group showed loose and thin granulation tissue, less fibroblasts and more inflammatory cells. In converse, bilirubin-treated group, showed more fibroblast proliferation, more blood vessels in perpendicular direction and few inflammatory cells. On day 14, the bilirubin-treated wounds showed thick, intact and fully formed epithelial layer, dense collagen fibers with parallel arrangement resembling normal skin and H & E score is significant higher from day 3 onwards (Fig. 2b) which represents better granulation tissue formation. On the other hand, there was thin, loose and immature epithelial layer, few inflammatory cells and less and loose collagen deposition was evident in control group.

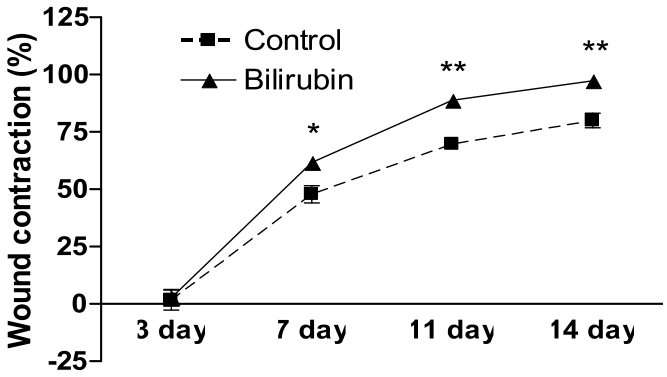

Fig.1b:
Per cent wound contraction in bilirubin-treated rats was greater, as compared to control on days 3, 7, 11 and 14 post-wounding. Data are expressed as mean ± SEM of 5 rats, *P<0.05, **P<0.005 versus control on the same day.

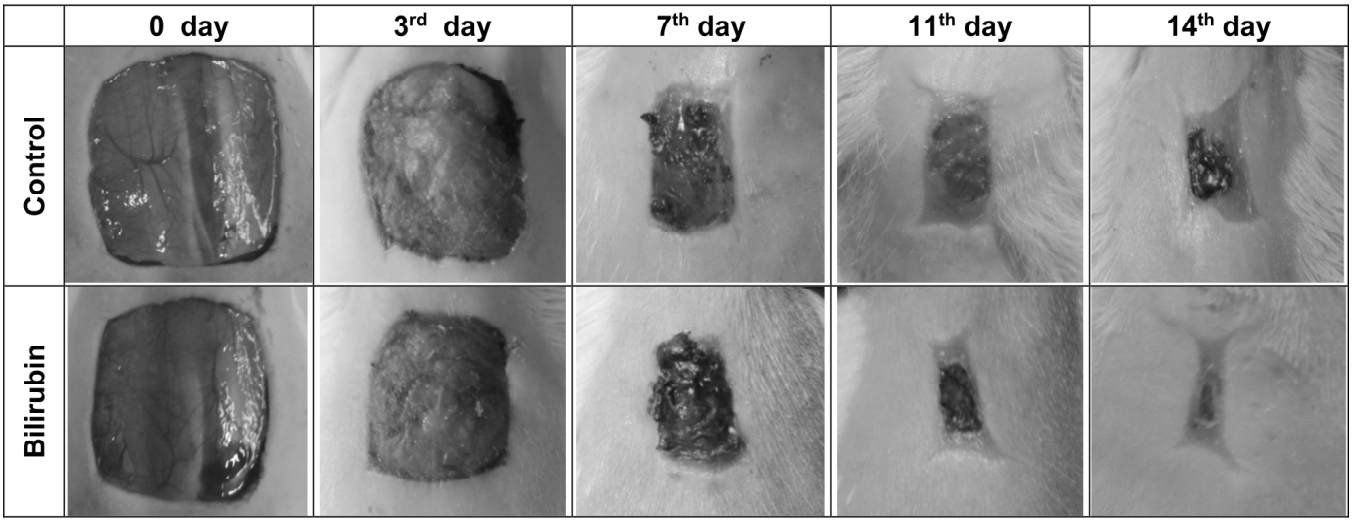

Fig. 1a:
Effect of bilirubin on gross appearance of healing wound and per cent wound contraction (calculated in respect to day 0) on days 3, 7, 11 and 14 post-wounding in rats.

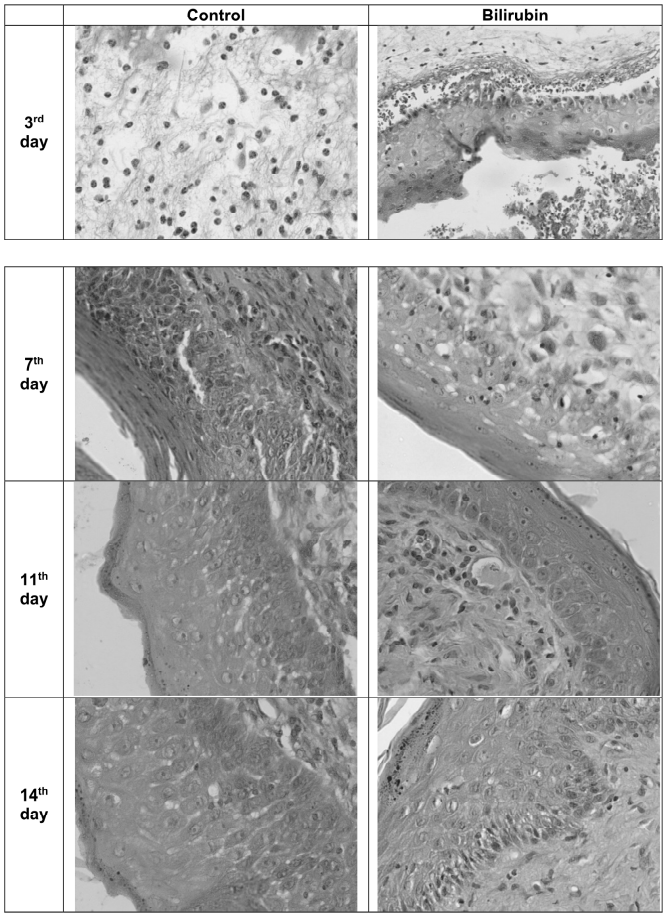

Fig. 2a:
Representative images of H&E stained histopathological sections of granulation tissues of control and bilirubin-treated rats (magnification 40x and scale bar 50 µm) on days 3, 7, 11 and 14 post-wounding.

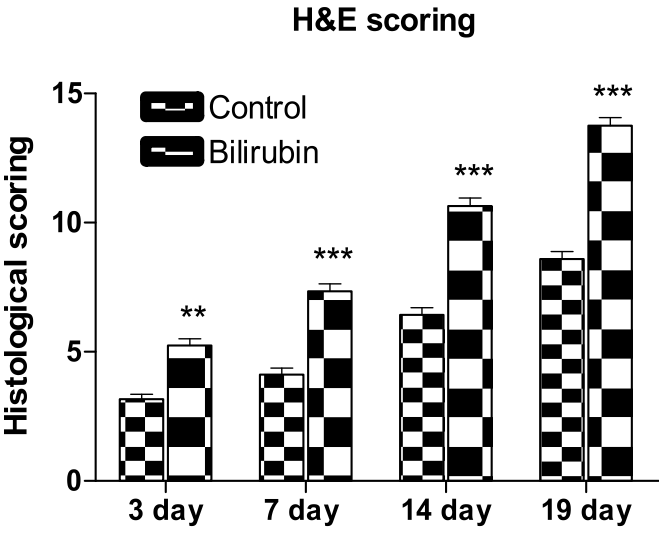

Fig. 2b:
Histological scoring of H&E stained granulation tissue sections (b) of control and bilirubin-treated rats on days 3, 7, 11 and 14 post-wounding. Data are expressed as mean ± SEM of 5 sections, $^{**}P<0.01$, $^{***}P<0.001$ versus control on the same day.

DISCUSSION

Wound healing is a process of synchronized interplay of a variety of cells, cytokines and growth factors, alteration of which leads to impairment of the healing process. Normal wound healing involves four temporally overlapping phases i.e. haemostasis, inflammation, proliferation and remodelling (Donald and Zachary, 2004). Bilirubin, the end product of heme catabolism, scavenges reactive oxygen species (ROS) *in vitro* and *in vivo*. In our earlier study, we have reported bilirubin to possess antioxidant and free radical scavenging property, as well as enhancing the activities of other antioxidants. Thus, bilirubin was shown to reduce oxidative stress during healing and accelerated wound healing (Ram *et al*., 2014). Bilirubin has also been shown to reduce mRNA expression of pro-inflammatory cytokine TNF-α and to increase the expression of anti-inflammatory cytokines IL-10 (Ahanger *et al.,* 2014) which facilitated wound healing. Here, in this study, we hypothesized that bilirubin enhance wound healing by the recruitment of several cells which are crucial for granulation tissue formation and its proper deposition during wound healing process. In the present study, Fig. 1b shows a significant increase in percent wound contraction in bilirubin-treated groups from day 7 to 14, as compared to control. The results indicate marked acceleration of wound healing process after bilirubin application and almost complete healing on day 14.

The wound gap is filled by the granulation tissue which in early stage are of immature type consisting of inflammatory cells, angioblasts, fibroblasts, collagen fibres, etc which in later stage becomes more matured and permanent and takes place in the gap formed by the formation of wound. The further maturity of such matured granulation tissue on the surface is responsible for wound closer, if it is not being completely covered by epidermal epithelial cells. The formation of granulation tissue is followed by two early events one is heamostasis and second is inflammation. More influx of inflammatory cells is prerequisite for early scavenging of irritant and tissue debris as well as release of growth factors for proliferation of fibroblast cells, angioblast cells and synthesis of matrix. This parameter has been used for comparison of effect of bilirubin histopathologically. Likewise, number of cells involved in formation of granulation tissue has also been taken into consideration for comparison. Moreover, early maturation of granulation tissue has been used as an important indicator for assessing the effect of bilirubin in promoting wound healing histopathologically, on the basis of, the fact that immature fibroblast cells are round, plump, while mature one is elongated and smaller in size (Werner *et al*., 2005). Likewise, more maturity of granulation tissue is also assessed by thin and loosely dispersed collagen fibres while in case of mature granulation tissue, they are found in wavy bundles and running parallel to each other

(Kant *et al*., 2013). Angiogenesis is closely related to the formation of granulation tissue and depends on the complex control system of proangiogenic and antiangiogenic factors produced by a variety of cells. Maximum capillary growth and differentiation occurs approximately between days 3-7 post-wounding during the proliferative phase of repair (Bao *et al*., 2009). In our study, formation of blood vessels from day 3 onwards and highest on day 11 and then decreased (Fig. 2). Once granulation is complete, angiogenesis ceases, as endothelial cells undergo apoptosis which is evident in histopathological findings.

One of the major components of extracellular matrix is collagen fibers which are responsible for the tensile strength of the wounds. Loosely arranged collagen fibers in the earlier stage of wound healing, which were replaced by a reorganized, more compact, thicker bundles and parallely arranged collagen fibres in the later stage of the healing process after bilirubin application in this study. VEGF stimulates migration of keratinocytes and fibroblasts (Stojadinovic *et al*., 2007), which in turn improve epithelialization and collagen synthesis. The increased total collagen fraction (both thicker and thinner collagen fibers) in the later stages of wound healing accelerates the progression of the wound towards maturity. More collagen fraction in the bilirubin-treated wounds reveals faster maturation of the wounds (Fig 2). Superior granulation tissue is characterized by the presence of fibroblasts with extracellular matrix (ECM) formation and well formed blood vessels in the perpendicular direction. In addition to the formation of ECM in healing tissue, its progressive degradation and remodeling in a regulated manner is essential to form mature healed wound tissue. Bilirubin-treated rats in this study showed formation of more fibroblast and collagen bundles in a time-dependent manner as evidenced in histopathological observations (Fig. 2), which indicates that bilirubin facilitated a well synchronized process of wound repair.

Thus, the results reveal that bilirubin treatment accelerated the timely progression of wound healing by migration of inflammatory cells, formation of blood vessels, deposition of keratinocytes, fibroblasts, collagen fibers and improved maturation of wound. In conclusion, bilirubin helped in healing process and proved its cutaneous wound healing potential in rats.

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Research Article

ANTI-NOCICEPTIVE ACTIVITY OF METHANOLIC EXTRACT OF SEABUCKTHORN (*HIPPOPHAE RHAMNOIDES*) PULP IN MALE WISTAR RATS

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ABSTRACT

In the present study anti-nociceptive activity of methanolic extract of *seabuckthorn (Hippophae rhamnoides) pulp* is evaluated in male wistar rats. Anti-nociceptive activity was evaluated by Hot plate, tail immersion & tail clip tests. Methanolic extract of Seabuckthorn pulp (MESBTP) was administered @ 250mg/kg & 500mg/kg body weight orally in all these tests. MESBTP @ 500mg/kg body weight to all the pain induced models showed significant antinociceptive activity. However, the effect was lower when compared with analgin. The results indicate antinociceptive activity of seabuckthorn pulp and support ethnomedical use of this plant in different painful conditions.

Key words: Hot plate test, Tail immersion test, Tail clip test

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) is a thorny nitrogen fixing deciduous shrub which is cultivated in various parts of the world for its nutritional and medicinal values (Oliver, 2001). Its fruit contain carotenes (a, b and d), Vitamins C and E, riboflavin, folic acid, tannins, sugar, glycerides of palmitic, stearic and oleic acids, polyphenols and some essential amino acids (Beveridge *et al.,* 1999). These compounds are of interest not only from the chemical point of view, but also because many of them possess biological and therapeutic activities including antioxidant, cardiovascular,cancer therapy, healing, anti-inflammation, antiradiation effect, treatment of gastrointestinal ulcers, as a liver protective agent, antioxidant, platelet aggregation,and immunomodulator (Cheng *et al.,* 2003). In the present study anti-nociceptive activity of methanolic extract of *seabuckthorn pulp* is evaluated in male wistar rats.

MATERIALS AND METHODS

Seabuckthorn leaves were collected from lahol and spiti region of Himachal Pradesh (India). Methanolic extract of *Seabuckthorn* pulp was prepared (MESBTP). A total of 24 Male Albino Wistar rats were used. The rats were housed under the standard environmental conditions and fed with standard pellet diet and water *ad libitum.* The rats were divided into four groups with six rats in each group. The group I served as control and received 0.5 ml of normal saline orally. Group II served as reference standard group to which Analgin (Novalgin,Aventis pharma) was given @ 200mg/kg body weight intraperitoneally. The groups III and IV received MESBT pulp @ 250mg/kg & 500mg/kg body weight orally. Analgesic activity was evaluated by Hot plate test; tail immersion test & tail clip test (Ghosh, 2005). In hot plate test, hot plate was maintained at 55±1^{0}C.The time taken to cause a discomfort reaction (licking paws or jumping) was recorded as response latency or reaction time. Before administration of the test compound or the standard, the normal reaction time was determined. The animals are submitted to the same testing procedure after 30, 60 and eventually 120 min after administration of the drug and test compound. For each individual animal the reaction time was recorded. A cut-off time of 30 seconds was followed to avoid any thermal injury to the paws. In Tail immersion test, tail of rat upto5cm was dipped into hot water maintained at 55±1^{0}C . Time taken by rat to flip its tail clearly out of water was taken as reaction time. The reaction time in seconds before administration of the drug was recorded for all the rats from each group. Reaction time in seconds after administration of drugs was recorded at an interval of 30, 60 and 120min. In tail clip test, clamp is applied at the distance of 1.5cm to the base of the tail and the time taken by the rat to remove the clip is recorded as the reaction time. The reaction time in seconds before administration of the drug was recorded for all the rats from each group. Reaction time in seconds after administration of drugs was recorded at an interval of 30, 60 and 120min. Statistical analysis was carried out using ANOVA followed by Dunnet's test. A 'p' Value < 0.05 was considered to be significant and 'p' Value < 0.01 was considered to be highly significant.

RESULTS

Analgin @ 200mg/kg body weight significantly ($p<0.05$) increased reaction time in tail immersion test from 60 min onwards (Table 2). Highly significant ($p<0.01$) increase in reaction time was observed by analgin from 60min onwards in tail clip and hot plate tests (Table 3,

Table 1). MESBTP @ 250mg/kg body weight significantly ($p<0.05$) increased the reaction time in tail immersion test at 120min of administration (Table 2). MESBTP @ 500mg/kg body weight significantly ($p<0.05$) increased the reaction time after 60 min of administration in tail clip and hot plate tests (Table 3, Table 1) and at 120min in tail immersion test (Table 2) .Highly significant ($p<0.01$) increase in reaction time was observed by MESBTP (500) at 120 min in hot plate and tail clip tests (Table1, Table 3).

DISCUSSION

Pain is an unpleasant sensation localized to a part of the body. The perception of pain is supported by a system of sensory neurons and neural afferent pathways that specifically respond to potentially noxious, tissue-damaging stimuli. The small diameter myelinated A-delta and the unmyelinated C fibres, present in nerves to skin and to deep somatic and visceral structures, respond maximally to painful stimuli. These are the primary afferent nociceptors (pain receptors) (Fields and Martin 2008). Inflammation produced by mild tissue damage or infection causes afferent C and A-delta fibres to be activated by low intensity stimuli and pain occurs (Dray, 1992). Substances like kinins, prostanoids, serotonin, histamine etc. are released upon damage to cells. These are potent algogenic substances and induce pain by directly stimulating nociceptors in skin, joints, muscles, as well as by sensitizing them to heat and mechanical stimuli (HLA and Nielson 1992).NSAIDs act primarily on peripheral pain mechanisms but also in CNS to raise pain threshold (Tripathi, 2008). They are the most commonly used anti-inflammatory, antipyretic, analgesic drugs. Most NSAIDs block prostaglandin synthesis by inhibiting COX-1 and COX-2 nonselectively, but now some selective COX-2 inhibitors have been developed. Of the common toxicities caused by NSAIDs due to inhibition of prostaglandin synthesis, gastric mucosal damage is most troublesome. This sometimes limits the use of this group of drugs in patients with chronic pain. Sea buckthorn (*Hippophae rhamnoides L.*) constitutes thorny nitrogen fixing deciduous shrub. Sea buckthorn is primarily valued for its very rich vitamins A, B1, B12, C, E, K, and P; flavonoids, lycopene, carotenoids, and phytosterols and therapeutically important since it is rich with potent antioxidants (Patel *et al.,* 2012). There are studies which have elicited gastro-protective effects of seabuckthorn, which may be further beneficial to patients who need analgesics. This study was thus done with the aim to assess the efficacy of seabuckthorn as an analgesic . A significant increase in the reaction time in the pain induced models indicated the analgesic effect by seabuckthorn pulp @ 500mg/kg body weight and also elucidates the involvement of central

Table 1:
Anti-nociceptive activity of MESBT pulp by hot plate method in rats.

Group	Dose(mg/kg)	Reaction time in seconds			
		pretreatment	30min	60min	120min
Control	0.5ml (NSS)	5.87± 0.62	5.65± 0.48	5.49± 0.51	5.53± 0.31
Analgin	200	6.55± 0.17	6.97±0.28	12± 0.85 **	9.6± 0.38**
MESBT pulp1	250	5.77 ± 0.28	5.91± 0.66	6.27 ± 0.70	7.5± 0.5
MESBT pulp 2	500	5.36 ± 0.28	5.79 ± 0.22	6.79± 0.58*	7.13 ± 0.27**

Data was analyzed by ANOVA Test followed by Dunnet's *post hoc* test. Each value is Mean ± SEM; n=6, *P<0.05, **P<0.01 when compared with pretreated group.

Table 2 :
Anti-nociceptive activity of MESBT pulp by tail immersion method in rats.

Group	Dose(mg/kg)	Reaction time in seconds			
		pretreatment	30min	60min	120min
Control	0.5ml	6.11 ± 0.63	6.34 ± 0.64	6.15 ± 0.93	6.1 ± 0.47
Analgin	200	5.67 ± 0.40	5.77 ± 0.95	8.97± 0.81*	8.74 ± 0.74*
MESBT pulp1	250	5.9 ± 0.57	6.32 ± 0.62	7.39 ± 0.51	8.39± 0.76 *
MESBT pulp2	500	6.17 ± 0.22	6.97 ± 0.33	7.48 ± 0.63	8.12 ± 0.55*

Data was analyzed by ANOVA Test followed by Dunnett's *post hoc* test. Each value is Mean ± SEM;

Table 3:Anti-nociceptive activity of MESBT pulp by tail clip method in rats.

Group	Dose(mg/kg)	Reaction time in seconds			
		pretreatment	30min	60min	120min
Control	0.5ml	6.45 ± 0.6	6.36 ± 0.46	6.22 ± 0.51	6.32 ± 0.27
Analgin	200	5.34 ± 0.32	6.52 ± 0.35	7.16 ± 0.44**	7.82 ± 0.32**
MESBT pulp1	250	5.79±0.2	5.9 ± 0.42	6.28 ± 0.53	7.08± 0.33
MESBT pulp2	500	5.4±0.3	6.51 ± 0.48	7.57 ± 0.77*	8.1 ± 0.4 **

Data was analyzed by ANOVA Test followed by Dunnet's *post hoc* test. Each value is Mean ± SEM;
n=6, *P<0.05, **P<0.01 when compared with pretreated group

mechanism in analgesic action. Analgesic effect mediated through central mechanism indicates the involvement of endogenous opioid peptides and biogenic amines like 5HT (Bensemana and Gascon 1978). Methanolic extracts of Sea buckthorn pulp @ 500mg/kg body weight to all the pain induced models resulted in significant increase in reaction time of rats. Hence it could be concluded that MESBTP has good efficacy as an analgesic. The potential of Seabuckthorn as an additive analgesic to conventional drugs may further be explored.

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Research Article

EFFECT OF CURCUMIN OINTMENT ON EXCISION WOUND HEALING BY MODULATING BIOCHEMICAL PARAMETERS IN WISTAR RATS

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ABSTRACT

Present study was conducted to explore wound healing potential of curcumin after topical application and its effect on body weight. Experimental rats were divided into group-I (vehicle-control), group-II (povidone-iodine) and group-III (curcumin). Excision wound was made in rats of all groups and 0.25g of vehicle, povidone-iodine (0.5%), and curcumin ointment (20%) had applied topically daily for 15 days to rats of group-I, II and III, respectively. Result of present finding showed curcumin improves biochemical parameters viz. hydroxyproline (29.58±1.82 *vs* 21.74±2.91 mg/g) and glucosamine (10.54±2.93 *vs* 5.61±1.78 mg/g) of healed skin of group-III rats compared to control. Similarly improvements in histo-morphology, wound contraction (day 13th 85.37±1.27 *vs* 72.76±9.64 %) and body weight gain was found in curcumin treated rats of group-III compared to control indicating curcumin possess wound healing potential seen in biochemical modulation and histopathological improvement.

Keywords: Curcumin, excision wound, topical-application, Wistar rats

INTRODUCTION

Skin restricts pathogens entry systemically and protects from environmental calamity. Wound is break in cellular and anatomical continuity of skin proceed to loss of functions (Prabu *et al*., 2008). Wound healing processes modify the cellular, biochemical and physiological activities followed by repair of connective tissues. In final stage of wound healing, skin restores its continuity and retrieves its loosed function (Bowler, 2004).

Curcumin is a yellow bioactive component of *Curcuma longa* which possess wound healing potential along with numerous other therapeutic potential (Raina *et al*., 2008; Rivera-Espinoza and Muriel, 2009). Therapeutic activities viz. anticancer, antiviral, antioxidant and anti-inflammatory of curcumin produced by modulating granulation tissue, transforming growth factor-β1 biosynthesis, and scavenging free radicals (Aggarwal and Harikumar, 2009; Mohanty *et al*., 2012).

Wound treatment includes either local application or systemically administration of drugs or both way of treatment followed together to minimize undesired consequences of wound repairing (Meyers *et al*., 1980). Wound healing can also be enhanced using active principles of plants like triterpenes, alkaloids, flavanoids and other biomolecules. In present study of wound management, active constituent curcumin was taken to analyze its impacts on wound repair, change of body weight, and speed of wound contraction.

MATERIALS AND METHODS

Experimental animals

Healthy adult Wistar rats weighing of 150-200g were procured from Indian Institute of Integrative Medicine (CSIR Lab), Jammu and randomly divided into three groups (group-I, II, & III) containing six rats in each. Rats were housed in polypropylene cages with free access of pelleted feed and water. A daily cycle of 12 h of light and similar 12 h of darkness was provided to animals. Institutional Animal Ethics Committee was permitted to conduct the experimentation.

Wound model

Overnight fasted rats were anesthetized using xylazine (10mg/kg) and ketamine hydrochloride (70mg/kg) intramuscularly. Dorsal part of inter-scapular region of anesthetized rats was swabbed with ethanol and shaved. At shaved region, an area of 2×2 cm^2 (400 mm^2) was marked using fabricated wooden block and excised to depth of loose subcutaneous tissue (Ahanger *et al*., 2011). Excised wound was made in all rats and divided into three groups namely group-I (vehicle-control), group-II (Povidone-iodine ointment) and group-III (curcumin ointment). About 0.25g of soft white paraffin, povidone-iodine ointment (0.5%) and curcumin ointment (20%) were applied topically over wound of group-I, II, and III rats daily for 15 day, respectively. After fifteen days of treatment period, on day 16th healed skin was removed to analyze biochemical parameters namely hydroxyproline (Woessner, 1961) and glucosamine (Rondle and Morgan, 1955) with little modification in protocol.

Histopathological changes

Healed skin was immediately fixed in 10% neutral buffered formalin solution. After 3-4 days of fixation, tissues

were washed in running water for 7 to 8 hrs, dehydrated in ascending grades of ethyl alcohol, cleared in benzene and embedded with melted paraffin wax. and further processed as per methods of Luna (1968).

Wound area measurement

Wound area was measured at predetermined interval on day 0, 4th, 7th, 10th and 13th. To measure wound area, rats were hold on table and a flexible transparent polyethylene sheet had placed over wound and margins marked with permanent marker (Ahanger *et al*., 2010). Wound area was expressed as per cent wound contraction using the formula (Ahanger *et al*., 2010),

$$\% \text{ wound contraction} = \frac{\text{Wound area paper weight - unhealed wound area paper weight}}{\text{Wound area paper weight}} \times 100$$

Statistical analysis

Results were subjected to analysis of variance (ANOVA) in completely randomized design (CRD) with statistical significance being tested using the Duncan Multiple Range Test (Snedecor and Cochran, 1989).

RESULTS

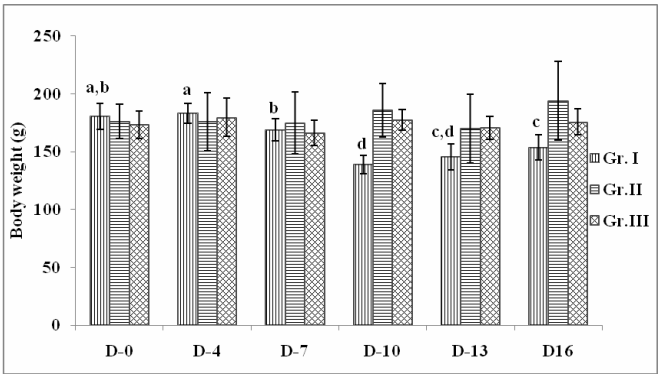
Fig.1 showing the effect of topical application of curcumin-ointment (20%) on body weight of Wistar rats (n=5). A significant decrease in body weight was found on day 7th (168.80±9.73g), day 10th (139.00±7.81g), day 13th (145.60±11.26g) and day 16th (153.60±10.85g) in comparison to day zero (180.60±11.26 g) within the group-I/control rats. However on day 10th highest fall in body weight was found in control rats. Although a non-significant change in body weight was found in rats of treated group-II and III.

Table.1 and Fig.2 depicts the effect of curcumin ointment on percentage wound contraction of excision wound in Wistar rats (n=5). On day 4th, a significant increase in wound contraction was found in curcumin treated rats of group-III as compared to vehicle control. On day 7th and 10th, a non-significant change in percentage wound contraction was found in both treated rats of group-II and III in comparison to control group-I. However, on day 13th, a significant increase in percentage wound contraction was found in rats of treated group-II and group-III in comparison to vehicle control.

Fig. 3 depicts the effect of curcumin ointment (20%) on hydroxyproline content of healed wound of Wistar rats (n=5). A significant increase in hydroxyproline content of healed wound was found in rats of both povidone treated (26.69±3.00mg/g) and curcumin treated (29.58±1.82mg/g) in comparison to control rats (21.74±2.91mg/g). However, a non-significant increase of hydroxyproline content of healed skin was found in curcumin treated rats (29.58±1.82mg/g) as compared to povidine treated rats (26.69±3.00 mg/g).

Fig. 4 depict the effect of curcumin ointment (20%) on glucosamine content of healed skin of rats (n=5). A significant increase of glucosamine content of healed skin was found in rats of both povidone treated (13.49±2.68mg/g) and curcumin treated (10.54±2.93mg/g) in comparison to control rats (5.61±1.78mg/g).

Slides of healed skin of rats of different groups are shown in Fig. A to H. To see the general morphology of healed skin haematoxylin and eosin (H&E) stain and for collagen fibers content of healed wound Van-Gieson's stain were used. Van-Gieson's stains tissue with different colour in which collagen appear bright red and nuclei blue, whereas cytoplasm, muscle, fibrin and red blood cells stain yellow. Intensity of red colour represents relative quantity of collagen fibers. H&E stains collagen fibers to pale pink, nuclei-blue, cytoplasm-purple and red blood cells cherry red. A significant increase in collagen fibers content indicates intense red colour (Fig. E&F) was found followed by angiogenesis, epithelialization and presence of



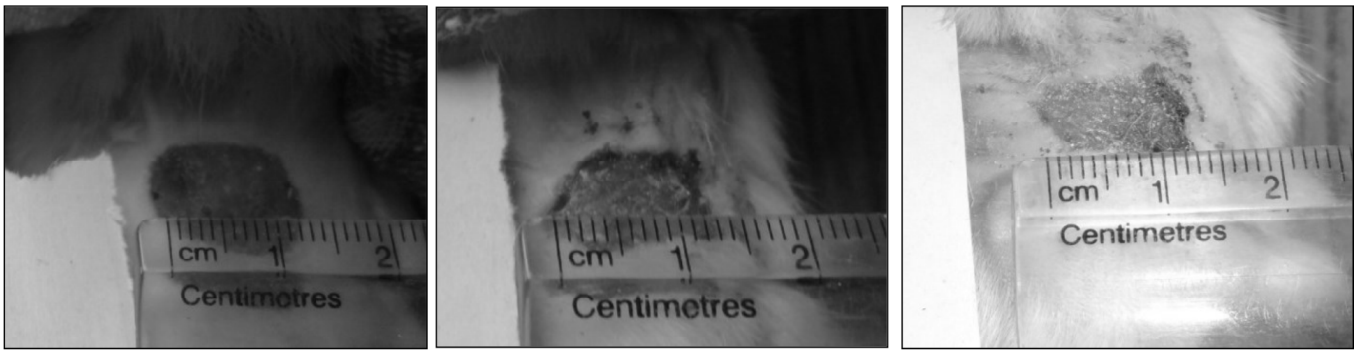
• D stands on day. Whereas superscript indicates level of significance (P<0.05) within the groups,
• Same superscript within the group indicates no significant variation,

Fig.1:
Showing the effect of topical application of curcumin-ointment (20%) on the body weight of Wistar rats (n=5).

Table. 1:
Showing the effect of topical application of curcumin-ointment (20%) on percentage-wound contraction in Wistar rats (n=5).

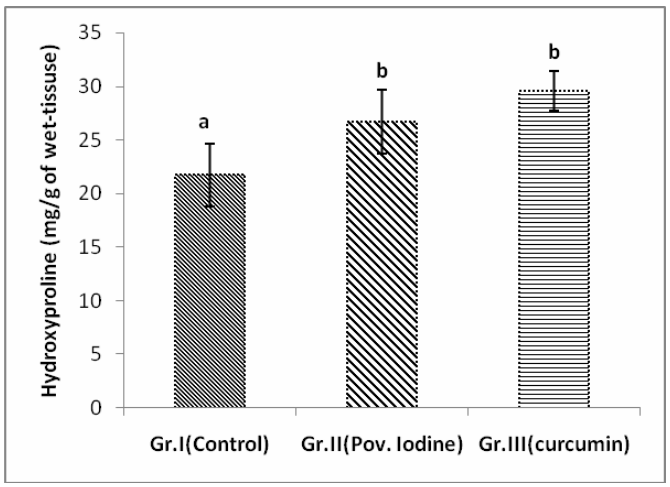
Group (s)	wound contraction (%) at different time-intervalValues (Mean±SD)			
	Day-4th	Day-7th	Day-10th	Day-13th
Group-I(Vehicle control)	24.74±3.61^{a}	55.39±3.38^{b}	67.83±8.62^{c}	72.76±9.64^{e}
Group-II(Povidone Iodine 0.5%)	25.77±1.08^{a}	58.91±5.46^{b}	76.02±4.29^{c}	88.31±2.26^{d}
Group-III(Curcumin 20%)	35.29±7.86^{b}	61.4±7.76^{b}	75.40±1.93^{c}	85.37±1.27^{d}

Different superscript vary significantly (P<0.05) between different groups on same day whereas same superscript indicates no-significant variation.



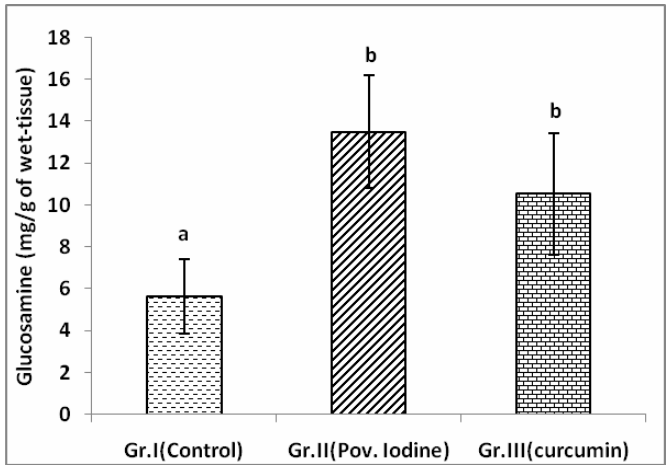
Group. I Group. II Group. III

Fig. 2:
Showing the effect of topical application of curcumin ointment (20%) on day 7th in Wistar rat



Different superscript vary significantly (P<0.05) between different groups, whereas same superscript indicates no-significant difference.

Fig.3:
Showing effect of topical application of curcumin-ointment on hydroxyproline-content of healed-skin of Wistar rats (n=5)



Different superscript vary significantly (P<0.05) between different groups, whereas same superscript indicates no-significant difference.

Fig.4:
Effect of topical application of curcumin-ointment (20%) on glucosamine content of healed skin of Wistar rats (n=5).

inflammatory cells (Fig. C&D) in healed wound of povidone (Fig. E&F) and curcumin (Fig. G&H) treated rats as compared to control (Fig. A&B).

DISCUSSION

Collagen and proteoglycan are ground substance of healing wound. Collagen being a component of connective tissue participates in structural framework of regenerating wound. Hydoxyproline and glucosamine are major constituents of collagen and proteoglycans, respectively (Panchatcharam *et al*., 2006). Hence hydroxyproline and glucosamine content of healed skin decide strength and speed of healing. In present study, curcumin significantly increases both hydroxyproline and glucosamine content of healed skin. Increase in content support significant increase of collagen, angiogenesis and inflammatory cells of curcumin treated healed skin showed in histopathological slide (Fig. G&H). Similar increase of collagen, inflammatory cells were also found in curcumin treated rats (Jagetia and Rajanikant, 2004; Mani *et al*., 2002; Panchatcharam *et al*., 2006). Increase of collagen content of healed wound either through early influx of fibroblast or by enhancing collagen deposition (Panchatcharam *et al*., 2006; Thangapazham *et al*., 2013). Curcumin improves wound contraction (%) by increasing pace of proliferation and early differentiation of fibroblast to myofibroblasts (Desmouliere *et al*., 1993). Similar improvements in wound contractions were reported by Interestingly, improvement in body weight of curcumin treated rat either through ameliorating stress condition or suppresses microbial load of wound (Ruby *et al*., 1995). Histopathological slide of healed wound revealed that curcumin significantly improves wound healing by increasing epithelialization, angiogenesis and collagen formation compared to control. Similarly, Kulac *et al*. (2013) also reveal similar improvements of histopathology of curcumin treated healed skin.

Present study showed that active constituent "curcumin" speed up wound healing what so ever are its routes of administration viz. topical/systemic. Rise in

Fig A-C:
Sections of healed wound of control group-I showing less deposition of collagen fibers and incomplete epithelization (A) ; less red colour indicating minimum deposition of collagen fibers (B) and epithelial hyperplasia indicating complete epithelization, deposition of collagen fibers, neo-vascularization and cellular infiltration of healed-wound of group-II (C) (H&E X100).

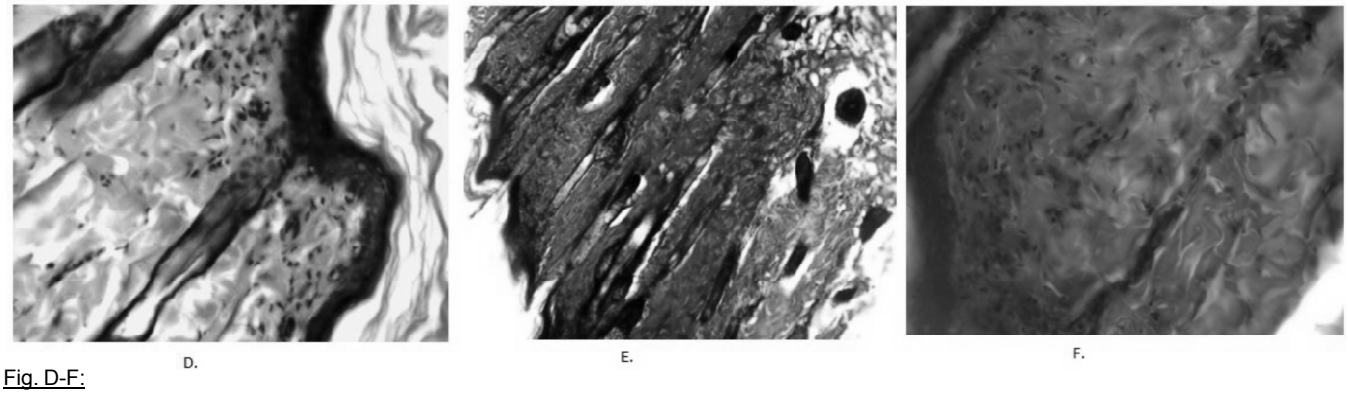


Fig. D-F:
Epithelial hyperplasia, deposition of collagen fibers, neo-vascularization and cellular infiltration (D)(H&E X400), epithelial hyperplasia indicating complete epithelization, more deposition of collagen fibers (red colour) of group-II (E) (Van-Gieson's stain X100) and epithelial hyperplasia, deposition of collagen fibers (red colour), neo-vascularization and cellular infiltration (F) (Van-Gieson's stain X400).

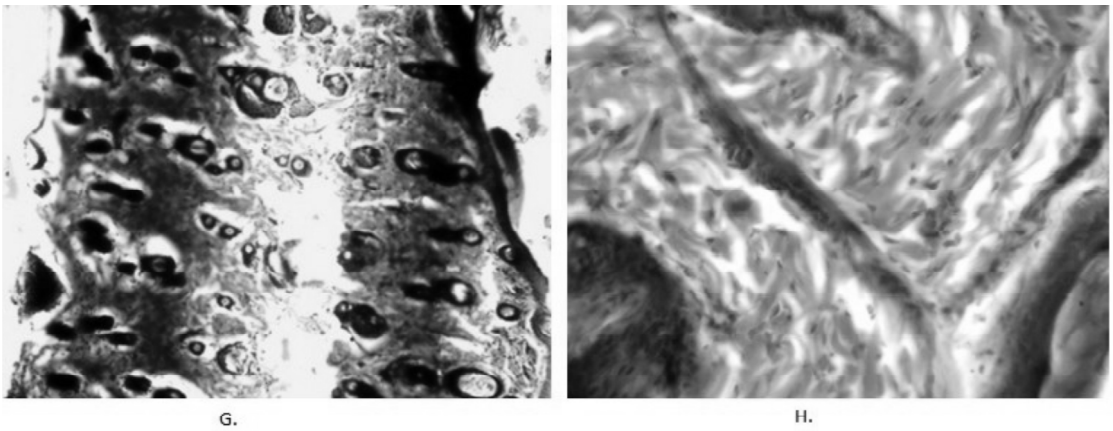


Fig. G-H:
Group-III showing epithelial hyperplasia and deposition of collagen fibers in (G) (H&E X100) and deposition of collagen fibers (red colour) (H) (Van-Gieson's stain X400).

hydroxyproline and glucosamine content of curcumin treated healed wound showing wound healing potency of curcumin. Further improvement of histopathology and health status of curcumin treated rats indicates role of curcumin in wound healing.

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Research Article

PHARMACOKINETICS AND INTRAMUSCULAR BIOAVAILABILITY OF KETOPROFEN IN PATANWADI SHEEP

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ABSTRACT

Pharmacokinetics and intramuscular bioavailability of ketoprofen, a non-steroidal anti-inflammatory drug was investigated in six female Patanwadi sheep after its single dose intravenous (IV) and intramuscular (IM) administration in a cross-over design study. Drug concentration in plasma was analyzed by HPLC (high performance liquid chromatography) and the data obtained were subjected to non-compartmental analysis. Following IV and IM administration of ketoprofen (3 mg/kg), values of elimination half-life (1.66 ± 0.12 and 3.31 ± 0.16 h), volume of distribution of drug at steady state (0.31 ± 0.01 and 0.83 ± 0.08 L/ kg) and total body clearance (331.60 ± 16.33 and 230.90 ± 17.81 mL/h/kg) were determined, respectively. The systemic (intramuscular) bioavailability of ketoprofen in sheep was 73.16 ± 5.58 per cent.

Key Words: Pharmacokinetics, Bioavailability, Ketoprofen, Patanwadi Sheep

INTRODUCTION

Ketoprofen [(RS)-2-(3'-benzoylphenyl) propanoic acid] (KTP) is a non-selective reversible cyclooxygenase (COX) inhibitor non-steroidal anti-inflammatory drug (NSAID). It is an aryl propionic acid derivative with two enantiomers *viz*. S(+) KTP and R(–) KTP (Sweetman, 2002). It is used as antipyretic, analgesic as well as anti-inflammatory in pneumonic, colic and arthritis diseases, and for the control of traumatic and postoperative pain in many species (Lees *et al*., 2004). Previously, pharmacokinetics of ketoprofen has been reported in ruminants (Singh *et al*., 2014). Intravenous (IV bolus) pharmacokinetics of KTP has also been studied in Corriedale-cross sheep (Ali *et al*., 2012), but intramuscular (IM) pharmacokinetics or bioavailability have not been reported. Moreover, intravenous (IV) pharamcokinetics was not studied in any Indian breed of sheep. Thus, present research work was carried out to establish pharmacokinetic parameters and intramuscular bioavailability of ketoprofen following IV and IM administrations at the dose rate of 3 mg/kg body weight in Patanwadi sheep.

MATERIALS AND METHODS

Animals

The study was conducted in healthy Patanwadi sheep housed in clean experimental pens and kept at Instructional Farm, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India. Six female sheep were selected randomly, weighing between 20 to 30 kg. The animals were housed two weeks prior to experiment for acclimatization, and all essential and standard managemental practices were followed to keep the sheep free from any stress. This study was prior approved by the Institutional Animal Ethics Committee (IAEC), Anand.

Experimental design and Sample Collection

Ketoprofen injection (Neoprofen, racemic mixture; 100 mg/mL; Vetnex Ranbaxy Fine Chemicals Limited, New Delhi) was administered both IV and IM at the dose rate of 3 mg/kg to each of six sheep with a 15 days washout period between administrations in a cross-over design manner. IV administration was done through left jugular vein, while IM injection was given in the gluteal muscles. Blood samples (3-4 ml) were collected into heparinized tubes from IV catheter (Venflon, 22 X 0.9 X 25 mm) fixed into the right jugular vein at 0 (pre-dosing), 2 min, 5 min, 10 min 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 12 h, 18 h, 24 h and 36 h respectively after IV administration. Similar time-points were used for sample collection following IM administration except for sample at 2 min. Plasma was separated after centrifugation (15 min, 2000*g*, 4^{0}C) of blood samples and stored at – 40^{0}C which were analyzed within 24 h.

Sample Preparation and Ketoprofen assay

Plasma KTP concentration was analyzed by HPLC method described by Wanwimolruk and Zoest (1991) with minor modifications. Method was standardized and validated in our laboratory before analysis of samples. Briefly, 100 µl of 4-N-HCL was added to plasma sample (1 mL) and mixed, then shaken for 10 min with 7 mL of diethyl ether. The mixture was centrifuged (10 min, 1500*g*, 4 ^{0}C) to separate the phases and the upper organic layer was transferred to a clean centrifuge tube containing 200 µl of 0.1 M sodium hydroxide. The mixture was shaken for 15 min and centrifuged (10 min, 1500*g*, 4 ^{0}C). Upper organic layer was discarded by aspiration and 50 µl of 0.5 M H_3PO_4 was added to the remaining aqueous phase. Then, the aqueous solution was transferred into a chromatographic vial; finally, 20 µl of

sample volume was injected into HPLC column.

Chromatographic conditions and validation

The HPLC apparatus (Lab Alliance, USA) comprised of quaternary gradient delivery pump (model AIS 2000) and UV detector (model 500) was used. Chromatographic separation was performed by using reverse phase C_{18} column (Thermo, 5 μ ODS; 250 × 4.6 mm ID) at room temperature. Mobile phase composed of a mixture of acetonitrile-methanol-water (25:25:50, v/v) containing 10 mM Na_2HPO_4, and adjusted to pH 4.15 with pure orthophosphoric acid. Mobile phase was filtered and pumped into column at a flow rate of 1.5 mL/min. The effluent was monitored through UV detector at 258 nm. The retention time of KTP was 10.4 min. Calibration curve was prepared by adding known amount of KTP to blank unfortified plasma for the expected range of concentrations from 0.05 to 50 μg/mL, processing such as samples exactly as described above. Quantification was done by reference to the resultant calibration curve. The calibration curve were prepared daily and not accepted unless it had a R^2 value > 0.99. The lower level of quantification (LLOQ) was 0.05 μg/mL. The mean recovery of KTP from plasma was ranging from 81.4 - 86.2 % for concentrations ranging from 0.05 - 50 μg/mL. The intraday and inter-day coefficients of variation (n=5) were satisfactory, with relative standard deviations (RSD) less than 10%.

Pharmacokinetic analysis

Pharmacokinetic parameters for KTP were determined for each sheep with commercial software (PK solution 2.0, USA) using non-compartmental approach of analysis. Following IM administration of the drug, maximum concentration (C_{max}) and time to reach the maximum concentration (T_{max}) were determined from the concentration-time curve. Bioavailability of the KTP was calculated using the following formula (Wagner, 1967).

$$F (\%) = [AUC (IM) \times t_{1/2\beta} (IV)] / [AUC (IV) \times t_{1/2\beta} (IM)] \times 100$$

Table 1:
Pharmacokinetic parameters of ketoprofen after single dose intravenous and intramuscular administration (3 mg/kg body weight) in Patanwadi sheep (n=6)

Parameters	Unit	Intravenous	Intramuscular
Cp0	μg/ mL	22.53 ± 1.43	——-
C_{max}	μg/mL	——-	4.93 ± 0.52
T_{max}	h	——-	0.50 ± 0.00
$t_{½\alpha}$	h	0.18 ± 0.01	——-
$t_{½\beta}$	h	1.66 ± 0.12	3.31 ± 0.16
AUC	μg.h/mL	9.32 ± 0.32	13.58 ± 0.91
AUMC	μg.h²/mL	9.33 ±0.98	50.57 ± 8.58
Vd_{area}	L/kg	0.80 ± 0.09	——
Vd_{ss}	L/kg	0.31 ± 0.01	0.83 ± 0.08
Cl_B	mL/h/kg	331.60 ± 16.33	230.90 ± 17.81
MRT	h	1.00 ± 0.06	3.67 ± 0.41
F	%	——-	73.16 ± 5.58

C_p0, theoretical plasma concentration of drug at zero-time; C_{max}, Maximum drug concentration; T_{max}, observed time for C_{max}; $t_{½\alpha}$, distribution half life; $t_{½\beta}$, elimination half life; AUC, area under plasma drug concentration-time curve; AUMC, area under first moment of curve; Vd_{area}, apparent volume of distribution; Vd_{ss}, volume of distribution at steady state; Cl_B, total body clearance; MRT, mean residence time; F, bioavailability.

RESULTS

The mean plasma concentration-time curves for KTP after single dose IV and IM administration (3 mg/kg) in Patanwadi sheep are presented in Fig. 1 as a semilogarithmic plot. Following IV administration, plasma drug concentration of 33.23 ± 1.72 μg/mL was achieved at 2 min which rapidly declined to 4.08 ± 0.20 μg/mL at 30 min. Thereafter, the KTP concentration in plasma diminished gradually and was not detectable after 8 h. High distribution rate constant (3.83 ± 0.14 /h) and low elimination rate constant (0.43 ± 0.03 /h) were observed. The mean distribution half-life ($t_{½\alpha}$), elimination half-life ($t_{½\beta}$), volume of distribution at steady state (Vd_{ss}), area under plasma drug concentration-time curve (AUC), and total body clearance (Cl_B) were 0.18 ± 0.01 h, 1.66 ± 0.12 h, 0.31 ± 0.01 L/kg, 9.32 ± 0.32 μg.h/mL and 331.60 ± 16.33 mL/h/kg, respectively.

Following IM administration, mean peak plasma concentration (C_{max}) of KTP (4.93 ± 0.52 μg/mL) was achieved at 0.50 h (T_{max}). The mean area under curve (AUC), elimination half-life, total body clearance and systemic bioavailability (F) were 13.58 ± 0.91 μg.h/mL, 3.31 ± 0.16 h, 230.90 ± 17.81 mL/h/ kg and 73.16 ± 5.58 %, respectively. Various pharmacokinetic parameters following IV and IM administration of the drug are presented in the Table 1.

DISCUSSION

Higher value of distribution rate constant (3.83 ± 0.14 /h) and lower distribution half life (0.18 ± 0.01 h) in present study indicate that KTP is rapidly distributed from circulation pool after IV administration, whereas lower elimination rate constant (0.43 ± 0.03 /h) indicates its slower elimination. Following single dose IV administration, the elimination half-life (1.66 h) of KTP is similar as observed in Pakistani sheep (1.91 h) (Ali *et al*., 2012) and goats (1.21 h) (Pranvendra *et al*., 2005) but more rapid elimination of drug has been reported in corriedale-cross sheep (0.86 h) (Landoni *et al*., 1999) whereas longer half-life (3.58 h) has been reported in buffalo calves (Rehman *et al*., 2013). Such variations in elimination half-life indicates interbreed and inter-individual variation of ketoprofen pharmacokinetics in sheep. Values reported for volume of distribution at steady state (0.31 ± 0.01 L/kg) following IV administration in present study is in accordance with values reported cows (Singh *et al*., 2014) where as slightly lower value reported for corriedale-cross sheep (Landoni *et al*., 1999) and higher value reported for buffalo calves (Rehman *et al*., 2013) and Pakistani sheep (Ali *et al*., 2012). The moderate volume of distribution following IV administration of KTP in cow calves in the present study is expected as

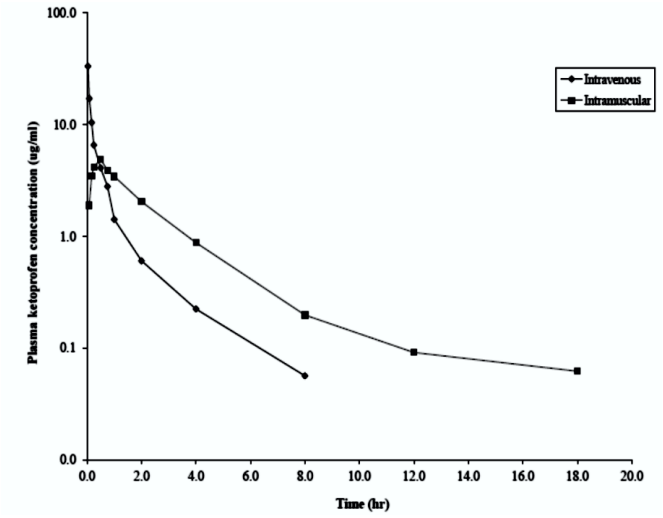

Fig. 1:
Semilogarithmic plot of ketoprofen concentration in plasma versus time following single dose intravenous and intramuscular administrations at the dose rate of 3.0 mg/kg of body weight in Patanwadi sheep. Each point represents mean ± S.E of six sheep

this drug is highly bound to plasma proteins, similar to most NSAIDs. The total body clearance (331.60 ± 16.33 mL/h/kg) following IV administration observed in the present study is in agreement with the reported values in an other study for both enantiomers of KTP in sheep (Landoni *et al*., 1999).

Following IM administration, longer elimination half-life of KTP (3.31 ± 0.16 h) was observed in the present study indicating that the drug being continuously absorbed during the elimination phase too.Slower clearance following IM administration (230.90 ± 17.81 mL/h/kg) was observed as compared to IV administration in present study which is close to values in goats.

The ultimate goal of a satisfactory dosage regimen of NSAIDs is to maintain the plasma drug level above median effective concentration [EC_{50}] for COX-2 enzyme during the course of treatment. Inhibitory activity of a NSAID on inflammatory exudate prostaglandin E_2 (PGE_2) is considered as the measure of COX-2 inhibition which was 0.012 µg/mL for KTP in sheep (Landoni *et al*., 1999). This concentration was maintained for up to 18 h following IM administration in present study. Therefore, ketoprofen given at the dose rate of 3 mg/kg body weight and repeated at 12 h would be satisfactory dosage regimen in sheep to achieve its therapeutic goals, as dosing interval of 18 h is not convenient. Longer elimination half-life, extensive volume of distribution at steady state and slower body clearance of KTP following IM administration as compared to IV administration makes it more suitable for intramuscular use in sheep. Good bioavailability and plasma concentration profile also supports intramuscular use of ketoprofen in Patanwadi sheep.

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Research Article

PROTECTIVE EFFECT OF *ZINGIBER OFFICINALE* EXTRACT IN GENTAMICIN INDUCED NEPHROTOXICITY IN WISTAR RATS

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ABSTRACT

The study was conducted to evaluate the protective effect of ethanolic extract of *Zingiber officinale Rosc* rhizome on gentamicin induced nephrotoxicity in Wistar rats. A control group (Group I) was compared with rats administered with 40 mg/kg gentamicin, once daily (Group II) and with rats administered ethanolic extract of rhizome of *Zingiber officinale Rosc* at dose level of 200 mg/kg along with 40 mg/kg gentamicin (Group III). The levels of blood urea nitrogen (BUN), Serum creatinine and uric acid were significantly increased in rats exposed to gentamicin alone (Group-II). Moreover, administration of gentamicin resulted in damage of kidney structures. Administration of ethanolic extract of rhizome of *Zingiber officinale Rosc* before gentamicin exposure prevented severe alterations of biochemical parameters and disruptions of kidney structures. In conclusion this study obviously demonstrated that pretreatment with ethanolic extract of rhizome of *Zingiber officinale Rosc* significantly attenuated the biochemical alterations induced by gentamicin.

Key words: *Zingiber officinale Rosc*, gentamicin, nephrotoxicity, Wistar rats.

INTRODUCTION

Amongst the various therapeutic agents which can cause nephrotoxicity, aminoglycoside antibiotics are the major cause. Aminoglycoside is the class of antibiotic which consists of gentamicin, streptomycin, neomycin, amikacin, kanamycin, tobramycin and netilmicin. Despite nephrotoxicity and ototoxicity, the aminoglycosides are continuously being used in clinical practice because of their bactericidal efficacy, synergy with β-lactam antibiotics, low cost and limited bacterial resistance. Gentamicin is an aminoglycoside antibiotic derived from *Micromonospora purpurea.* It inhibits the growth of a wide variety of gram-positive and gram-negative microorganisms, including strains resistant to tetracycline, chloramphenicol, kanamycin, and colistin; particularly strains of *Pseudomonas, Proteus, Staphylococcus and Streptococcus.*

Zingiber officinale Rosc, commonly known as ginger belongs to family *Zingiberaceae* is originated in South East Asia. The underground stem or rhizome of this plant has been used as a medicine in Asian, Indian and Herbal traditions since ancient times. Rhizome is stout, tuberous, horizontal consisting of many persistent roundish joints (Kirtikar *et al*., 1993 and Bentley *et al*., 1983). One of the active principle of ginger extract 6–gingerol exhibits potential renoprotective activity against cisplastin induced acute renal failure in rats (Kuhad *et al*., 2006). The nephroprotective effect of rhizome extract of *Zingiber officinale* Rosc in gentamicin induced nephrotoxicity in Wistar rats was therefore studied.

MATERIALS AND METHODS

Preparation of extract of *Zingiber officinale Rosc*

Rhizome of *Zingiber officinale* Rosc were sliced into slices with home slicer and dried under shade and then pulverized into fine powder by using mixer grinder. Ethanolic extract of rhizome of *Zingiber officinale* Rosc was prepared using Soxhlet apparatus and extractability percentage was determined as per the method suggested by Rosenthaler (1930).

Experimental Design

The total numbers of 36 Wistar rats of either sex were procured from Haffkkin institute, Mumbai, Maharashtra for the present investigation. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC). Rats were divided randomly into 3 groups (Table 1). Group I served as a control while in Group II rats were injected with gentamicin sulphate @ 40 mg per kg body weight I/P daily for 14 days. In Group III rats were given ethanolic rhizome extract of *Zingiber officinale* Rosc @ 200 mg per kg body weight orally as a preventive regimen along with the injection of gentamicin sulphate @ 40 mg per kg body weight I/P for the period of 14 days.

Phytochemical studies

Phytochemical studies were carried out as per standard method (Prabhuji *et al.,* 2005). Ethanolic extract of rhizome of *Zingiber officinale* Rosc was subjected to series of phytochemical tests for the presence of alkaloids, glycosides, proteins, reducing sugar, tannins, resins, sterols, phenolic compounds, saponins, flavonoids and anthraquinones.

Biochemical studies

All above treatments were given for 14 days, after which all the rats were sacrificed. Kidneys were collected for relative organ weight for histopathological evaluations. Blood samples from all the animals were collected for

biochemical estimations of blood urea nitrogen (BUN), creatinine and uric acid on day 0 (pre-treatment) and day 14 (post-treatment).

Statistical analysis

The data collected for various parameters were statistically analyzed by using analysis of variance (ANOVA) as per Panse and Sukhatme (1969) and Snedecor and Cocharan (1994). All the values in the text were expressed as mean ± SE

RESULTS AND DISCUSSION

The various experimental investigations recorded were phytochemical analysis of ethanolic extract of rhizome of *Zingiber officinale* Rosc, group wise mean body weight, relative organ weight and biochemical investigations.

The qualitative tests conducted for the presence of different phytochemicals of ethanolic rhizome extract of *Z. officinale* Rosc showed the presence of alkaloids, glycosides and flavonoids.

There was significant (Pd"0.01) increase in b wt in group I from the day 0 (pre-treatment) to day 14 (post treatment). However, there was significant decrease in b wt of group II. In preventive regimen, it was further noticed that there was significant (Pd"0.05) recovery in b wt in group III from day zero to 14(Table 2). The animals of Group II showed significant decrease in body weights due to adverse effect of gentamicin treatment. The treatment with Rhizome extract in group III improved the body weight indicating that *Z. officinale* rhizome extract had significantly ameliorated the effect of gentamicin.

As shown in Table 3, it was noticed that the mean BUN level, creatinine and uric acid in animals of the control Group I remained unchanged from day 0 to day 14. However, it was found that gentamicin treatment significantly (Pd"0.01) increased the BUN level, creatinine and uric acid on day 14 in Group II and III indicating the severe kidney damage which was also evidenced by histopathological examination. However, the preventive regimen dose of *Z. officinale* Rosc rhizome extract prevented the increase in the levels of BUN, creatinine and uric acid in Group III on day 14 as compared to in Group II indicating nephroprotective effect of *Z. officinale* Rosc rhizome extract. The results of the present study were in agreement with Nale *et al*. (2012) and Ruby *et al*. (2011).

Postmortem examinations of all the rats sacrificed during the experiment were performed and gross lesions if any were noted. Grossly, no appreciable lesions were observed in any of the organ in the rats of all the treatment groups. Gentamicin induced the formation of reactive oxygen species such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) from renal mitochondria that leads to nephrotoxicity (Mazzon *et al.,*

Table 1:
Experimental groups

Group	No. of rats	Treatment
I	12	Control (Negative control)
II	12	Gentamicin sulphate @ 40mg/kg b.wt. I/P for 21 days (Positive control)
III	12	Gentamicin sulphate @ 40mg/kg b.wt. I/P + ethanolic extract of rhizome of *Zingiber officinale* Rosc. (ZOEE) @ 200mg/kg b.wt. P.O. for 14 days (preventive regimen)

Table 2:
Effect of *Z. officinale* Rosc rhizome extract on body weight (g) and relative kidney weight (%) in different groups of gentamicin induced nephrotoxic and control rats.

Group	Body weight (g)		Relative kidney weight (%)
	Day 0(pre-treatment)	Day 14(post-treatment)	onDay 14(post-treatment)
Group I	221.42 ± 5.99[a]	261.87 ± 5.82[a]	0.340 ± 0.011[c]
Group II	219.00 ±5.58[a]	216.93 ± 5.57[b]	0.517± 0.016[a]
Group III	223.67 ± 6.82[a]	263.27 ± 6.20[a]	0.387 ± 0.018[b]

Means bearing different superscripts within the same column differ significantly (P<0.05)

Table 3:
Effect of *Z. officinale* Rosc rhizome extract on blood urea nitrogen (BUN) (mg/dl), creatinine (mg/dl) and uric acid (mg/dl) in gentamicin induced nephrotoxic rats.

Group	Biochemical parameters					
	BUN (mg/dl)		Creatinine (mg/dl)		Uric acid (mg/dl)	
	Day 0 (pre-treatment)	Day 14 (post-treatment)	Day 0 (pre-treatment)	Day 14 (post-treatment)	Day 0 (pre-treatment)	Day 14 (post-treatment)
I	17.33 ± 0.36[a]	17.50 ± 0.28[c]	0.615 ± 0.013[a]	0.605 ± 0.016[c]	3.02 ± 0.10[a]	3.05 ± 0.09[b]
II	17.72 ± 0.40[a]	24.82 ± 0.43[a]	0.625 ± 0.014[a]	1.128 ± 0.047[a]	2.84 ± 0.08[a]	5.03 ± 0.40[a]
III	16.99 ± 0.25[a]	19.07 ± 0.78[b]	0.633 ± 0.019[a]	0.775 ± 0.044[b]	2.89 ± 0.09[a]	3.12 ± 0.13[b]

Means bearing different superscripts within the same column differ significantly (P<0.05)

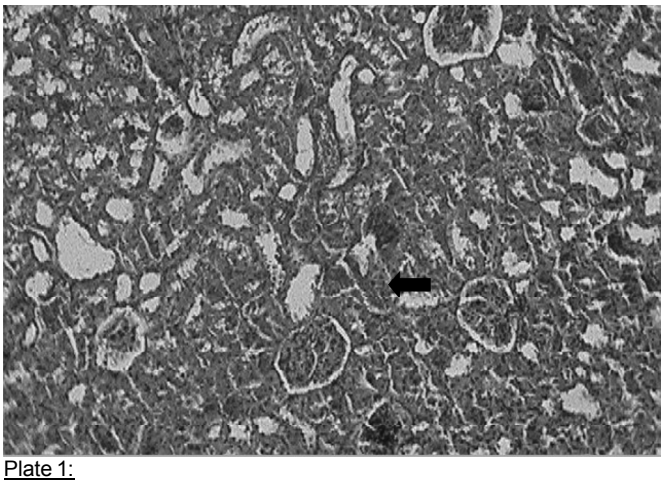

Plate 1:
Photomicrograph showing normal histological features of renal tubule and glomeruli.100 X H&E

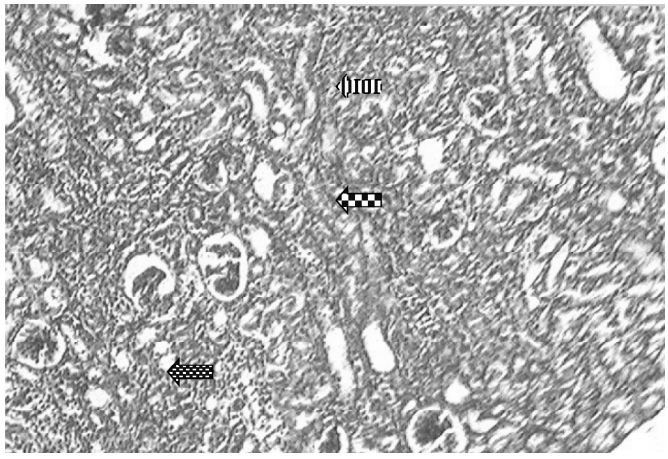

Plate 2:
Photomicrograph showing cellular infiltration (arrow), glomerular necrosis and glomerulopathies (arrow), interstitial nephritis (arrow), tubular degeneration and necrosis (arrow), 100X H&E stain pathological grade - ++++ (Top to bottom)

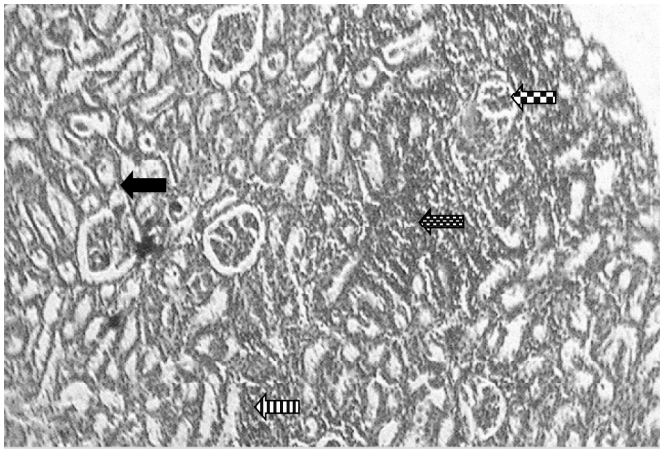

Plate 3:
Plate 3: Photomicrograph showing glomerular necrosis and glomerulopathies (arrow)tubular degeneration and necrosis (arrow), interstitial nephritis (arrow) cellular infiltration (arrow), 100X H&E stain pathological grade - ++ (Top to bottom)

2001).

Result of this study confirmed that gentamicin at a dose of 40 mg/kg resulted into nephrotoxicity in Wistar rats and was characterized by decrease in body weight and increased kidneys weight, BUN, serum creatinine and uric acid values. The mechanism involved in exhibiting nephroprotective activity might be due to antioxidant activity of phytoconstituents of *Z. officinale* Rosc rhizome extract which may act as free radical scavengers that restored the gentamicin induced oxidative stress in animals. Further detailed studies are required to understand precise mechanism of nephroprotective activity and isolation and identification of nephroprotective fractions of *Z. officinale* Rosc rhizome extract on molecular basis.

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Research Article

EVALUATION OF HEMATOLOGICAL ALTERATIONS ON SUB ACUTE EXPOSURE OF SPINOSAD IN MICE

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ABSTRACT

Spinosad was administered intraperitoneally to Swiss albino male mice in two different doses (29 mg/kg and 58 mg/kg body weight) for a period of 28 days. TLC was significantly higher in both the treated groups. The absolute count of leucocytes showed that neutrophil count and lymphocyte count increased at both dose levels with neutrophil count showing significant increase at higher dose. TEC did not show any biologically meaningful difference at any of the dose levels. Hb and Hct decreased in a dose dependent manner. At lower dose MCV value decreased significantly but at higher dose level, both MCV and MCH values decreased highly significantly suggesting spinosad toxicity caused microcytic hypochromic anaemia.

Keywords: Spinosad, haemoglobin, mice.

INTRODUCTION

Spinosad is a new class of insecticides, used to control a variety of insect pests. It is a mixture of two most active naturally occurring metabolites (spinosyns A & D). It has an excellent environmental and mammalian toxicological profile (Thompson *et al.*, 2000). Blood parameters are probably the more rapid and detectable variations under stress and are fuel in assessing the health conditions (Hymavathi and Rao, 2000). The complete blood count (CBC) can be used to monitor the severity of manifestations of illness. For this reason, this study was planned to investigate the effect of sub-acute doses of formulated spinosad on the hematology of Swiss albino male mice.

MATERIALS AND METHODS

Experimental animals

Swiss albino male mice weighing 20-25g were procured from Disease Free Small Animal House, LLR University of Veterinary and Animal Sciences (LUVAS), Hisar. The experimental animals were housed in polyacrylic cages, in groups of six animals per cage, in the Departmental Small Animal House. The animals were kept at room temperature with a natural light-dark cycle and provided with feed and tap water *ad libitum* and acclimatized for 5 days before the experiments. The prior approval of IAEC for the protocol of this study was obtained. The formulation product of spinosad, Tracer (Spinosad 45% SC, Dow AgroSciences India Pvt. Ltd.) was used in this study.

The hematological alterations induced by spinosad were studied in mice at two dose levels of 1/20th (5%) and 1/10th (10%) of MTD (MTD from preliminary studies was determined to be 580 mg/kg i.p. and thus the selected doses were 29 mg/kg and 58 mg/kg; i.p. of spinosad, respectively).

Experimental design

Each dose of the insecticide was administered daily to a group of 6 male mice i.p. for a period of 28 days. The results were compared with the control group of 6 male mice receiving normal saline @ 10 ml/kg body weight, i.p. daily for 28 days. Blood from individual animal was collected from retro-orbital plexus at the time of sacrifice using dry sterilized vials containing anticoagulant, sodium EDTA. Various haematological parameters viz. haemoglobin (Hb), hematocrit (Hct), total erythrocyte count (TEC), total leukocyte count (TLC), absolute leukocyte count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count were determined using automated hematology analyzer.

Statistical analysis

Data were analysed by one-way ANOVA followed by Dunnet's multiple comparison test. Results were expressed as mean ± standard error of mean. The threshold of statistical significance was set at p d" 0.05. Statistical analysis was performed using Graph pad prism version 5.03.

RESULTS AND DISCUSSION

The effect of treatment of spinosad on blood parameters in Swiss albino male mice is presented in Table 1. The TLC values in both the treated groups were significantly higher than the vehicle control group. The absolute count of leucocytes showed that neutrophil count and lymphocyte count increased at both dose levels as compared to saline treated group, with neutrophil count showing significant increase at higher dose. However monocyte, eosinophil and basophil count did not show significant difference from saline treated group. However, Hb and Hct values decreased in a dose dependent manner in spinosad treated groups. The hematocrit percent

significantly decreased in higher dose group as compared to saline treated group. Also, spinosad treatment caused significant increase in platelet concentration at both doses. Similarly, MCV and MCH values decreased in a dose dependent manner in spinosad treated groups. At lower dose MCV value decreased significantly but at higher dose level, both MCV and MCH values decreased highly significantly as compared to saline treated group. However MCHC values did not show any biologically meaningful difference in spinosad treated groups compared to the saline treated group.

Absolute lymphocyte and neutrophils count increased at both dose levels of spinosad treatment as compared to saline treated group. However, absolute monocyte, eosinophil and basophil count did not show significant differences from saline treated group. Moreover, neutrophil count was significantly higher at higher dose levels of spinosad as compared to saline treated group. Similar increase in TLC has been reported by Mansour *et al*. (2007) in rats given spinosad orally for 21 days and Zidan and Galal (2012) in rats feeding for 90 days on wheat grains treated with two different concentrations of malathion and spinosad. Such increase of WBC's may be due to the activation of the animal's defence mechanism and the immune system causing stimulated lymphopoiesis and/ or enhanced release of lymphocytes from lymph myeloid tissue (Das and Mukherjee, 2003). Such lymphocyte response might be due to the presence of toxic substances which induce tissue damage and severe disturbance of the non-specific immune system leading to increased production of leukocytes. The observed effects of spinosad insecticide, which represented by increase of total leukocyte counts (TLC), lymphocyte and neutrophil count in the blood of the treated mice, are generally in agreement with the results of several investigations. Yano *et al.* (2002) reported that after rat exposure to spinosad, white blood cell count of females from the 0.1% group was 39% higher than the controls after 18 months and noted that this difference was likely related to inflammation of the lung and thyroid gland in these rats. Stebbins *et al.* (2002) found that WBC's counts of male mice and females given 0.036% spinosad and females given 0.024% spinosad, were 2 - 2.5 times higher than the controls after 12 months and noted that the higher WBC's counts were likely related to inflammation of the stomach observed in these mice.

In the present investigation, RBC count did not show any biologically meaningful difference at any of the dose levels of spinosad as compared to saline treated group. However Hb and Hct decreased in a dose dependent manner in spinosad treated groups. Also, spinosad treatment caused significant increase in platelet concentration at both doses. Similarly, MCV and MCH values decreased in a dose dependent manner in spinosad treated groups suggesting spinosad toxicity caused microcytic hypochromic anaemia. In general, anaemia; reduction of haemoglobin in the blood can reflect impaired synthesis of haemoglobin (e.g. in iron deficiency) (Murray *et. al*., 2007). Many laboratories have reported the induction of anaemia with experimental insecticidal exposure of animals (Ali and Shakoori, 1990). Also, the hepatic heme biosynthesis has already been reported to be affected by insecticidal exposure, which also contributes to decreased Hb (Taljaard *et al*., 1972). Our findings are in agreement with the results reported by Yano *et al.* (2002), who found that male rats given 0.2% spinosad for 13 weeks had significant decreases in Hb concentration (60%) relative to control. The authors referred the observed anaemia to the decrease in Hb synthesis. In this study, the reduction in haemoglobin concentration may be attributed to the toxic effect of pesticides on bone-marrow and/or to decreased heme synthesis. Stebbins *et al.* (2002) reported

Table 1:
Effect of spinosad on total leukocyte count (TLC) and different leukocyte count (x10³/iL) in Swiss albino male mice

Parameters	Saline @10 ml/kg	Spinosad@ 29mg/kg	Spinosad@58 mg/kg
Total Leukocyte Count (x10³/iL)	4.33 ± 0.06	10.89 ± 1.39*	11.66 ± 1.68*
Neutrophil (x10³/iL)	1.81 ± 0.20	5.65 ± 0.96	7.25 ± 0.98*
Lymphocyte (x10³/iL)	2.73 ± 0.49	4.92 ± 0.61	3.96 ± 0.71
Monocyte (x10³/iL)	0.10 ± 0.02	0.20 ± 0.06	0.27 ± 0.06
Eosinophil (x10³/µL)	0.01 ± 0.01	0.08 ± 0.03	0.12 ± 0.03
Basophil (x10³/µL)	0.06 ± 0.04	0.04 ± 0.01	0.06 ± 0.01
TEC (x10⁶/iL)	8.39 ± 0.66	8.52 ± 0.31	8.53 ± 0.23
Hb (g/dL)	12.73 ± 1.12	12.38 ± 0.39	10.98 ± 0.35
HCT (%)	41.07 ± 3.04	39.44 ± 1.60	35.05 ± 0.95*
Platelet (x10³/iL)	711 ± 101.69	1149 ± 111.67*	1416.50 ± 91.74**
MCV (fL)	49.03 ± 0.32	46.26 ± 0.67*	41.08 ± 0.33**
MCH (pg)	15.20 ± 0.25	14.54 ± 0.22	12.90 ± 0.20**
MCHC (g/dL)	30.93 ± 0.68	31.44 ± 0.39	31.33 ± 0.36

Significant (P<0.05) difference thaw vehicle counts

that erythrocytic parameters (RBC's count and haemoglobin concentration) were decreased approximately 10 - 20% in male mice given 0.036% spinosad after 3 and 12 months. It was inferred that these changes were due to an increased rate of breakdown of red cells and/or the toxic effect of pesticides on bone-marrow. The reduction in MCH may be due to destruction of RBC (size and shape) and decrease in Hb synthesis and haemoglobin content. These symptoms imply the microcytic hypochromic anaemia.

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Research Article

EFFECT OF SUBSTANCE P ON THE EXPRESSION OF HIF-1A AND SDF-1A, AND ANTIOXIDANT ENZYMES LEVELS IN THE WOUND TISSUE OF DIABETIC RATS

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ABSTRACT

Impaired vascularization and increased oxidative stress due to persistent inflammation in the diabetic wound delay the healing process. Substance P (SP) increases microvessel density and has potential to cause sharp acute inflammation. So, present study was conducted to investigate the effect of exogenous SP on the vasculogenesis in diabetic wound site by assessing the expression of hypoxia-inducible factor 1-alpha (HIF-1α) and stromal cell-derived factors-1alpha (SDF-1α), and antioxidant enzyme levels were measured for determining the oxidative stress state. The normal saline, pluronic gel and SP (10^{-6}M) were topically applied once a day on the wound of diabetic rats of control, gel- and SP-treated groups, respectively, for 19 days. The mRNA expression of HIF-1α was lower and of SDF-1α was higher in SP-treated wounds throughout the experiment in comparison to control. SOD levels were markedly higher in gel- and SP-treated groups on day 7. The levels of catalase and glutathione peroxidase were higher in SP-treated group from day 7 onwards in comparison to other groups. In conclusion, SP has the potential for vasculogenesis at wound site and cytoprotectant action, which makes it a very good agent for faster wound healing in complicated cases particularly where vasculogenesis is compromised.

Keywords: Diabetic rats; Wound healing; Substance P; HIF-1α; SDF-1α; vasculogenesis; antioxidant enzyme.

INTRODUCTION

Diabetes mellitus is a very complex disease in people and equally so in the dog and cat (Hoenig, 2002). The most common complications involved in a diabetic for delayed wound healing are: reduction in chemotactic and phagocytic activities of neutrophils (Alba-Loureiro *et al.,* 2006), decreased angiogenesis, decreased vasculogenesis due to decreased number of endothelial progenitor cells (EPC) (Loomans *et al.,* 2004), decreased endothelial nitric oxide synthase (eNOS) activity (Gallagher *et al.,* 2007), depletion of substance P (SP) in the central and peripheral system (Kunt *et al.,* 2000), increased oxidative stress (Soneja *et al.,* 2005) and reduced number of growth factors (Beer *et al.,* 1997).

Neovascularization of the wound's granulation tissue arises by the processes of angiogenesis (formation of blood vessels from preexisting ones) and/or vasculogenesis (formation of blood vessels from endothelial progenitor cells) (Bauer *et al.,* 2005). Within the wound, neovascularization occurs because of local factors that stimulate adjacent cells (angiogenesis) and because of recruited circulating bone marrow derived (BMD) endothelial progenitor cells (EPC). Many factors have been identified which are involved in stimulation, promotion, and stabilization of new blood vessels (Johnson and Wilgus, 2012).

SP, an 11–amino acid neuropeptide, is a member of a family of tachykinins has shown its angiogenic potential in normal and diabetic wounds in our earlier studies, which caused the faster healing (Kant *et al.,* 2013; Kant *et al.,* 2015). HIF-1 is necessary for expression of multiple angiogenic growth factors and recruitment of endothelial progenitor cells for vasculogenesis (Ceradini *et al.,* 2004). HIF-1alpha pathway is an important pathway which plays a pivotal role in wound healing. So, the present study was conducted to investigate the effect of exogenous SP on the vasculogenesis by assessing the expression of HIF-1alpha and stromal cell-derived factors-1alpha (SDF-1α). Additionally, the antioxidant enzyme levels were measured for determining the oxidative stress state in the wound of diabetic rats.

MATERIALS AND METHODS

Experimental animals and diabetes induction

Adult healthy male Wistar rats of about 170–200 g were procured from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (U.P.), India. The experimental protocol was approved by the Institute Animal Ethics Committee. All the rats used in this study received humane care in accordance with National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH Publication N. 85-23, revised 1985). Single intraperitoneal injection of streptozotocin (60 mg/kg; Sigma-Aldrich, USA) was given to all the rats for the induction of Diabetes mellitus. After 48 hours, animals showing blood glucose levels e"300mg/dl were selected. The open excision-type wound 2×2 cm^2 (H"400 mm^2) was created on the back of sixty diabetic animals after 7 days

of diabetic induction under the pentobarbitone sodium (40 mg/kg) anesthesia. Wounded rats were housed individually in properly disinfected cages following recovery from anesthesia.

Animals were evenly divided in the following 3 groups:

1) Control: Sterile normal saline was topically applied on the wounds once daily for 19 days.

2) PF-127 gel-treated (gel-treated): 400 µl of PF-127 gel (25%, Sigma Aldrich, USA) was topically applied once daily for 19 days.

3) SP-treated: 400 µl of SP (10^{-6}M, Sigma Aldrich**)** in PF-127 gel (25%) was topically applied on wounds once daily for 19 days.

Tissue harvesting

On days 3, 7, 14 and 19, granulation/healing tissue was collected after killing five animals from each group. One portion of collected tissue was stored in RNA stabilization reagent (RNA*later*™, Qiagen, USA) at -20ºC until RNA extraction and the second portion was homogenized in ice-cold lysis buffer. The homogenized tissue was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was prepared and stored at -80ºC till further processing for enzyme linked immunosorbant assay (ELISA).

Real-time RT-PCR

The mRNA expressions of hypoxia-inducible factor 1-alpha (HIF-1α) and stromal cell-derived factors-1alpha (SDF-1α) were determined with real-time RT-PCR. RNA was isolated from wound tissues using RibozolTM RNA extraction reagents (Amresco, USA) and subsequently cDNA was synthesized using cDNA synthesis kit (Fermentas, Lafayette, CO, USA). cDNA was used as a template for the real time RT-PCR. The real time PCR assay was performed by using 2×QuantiTect SYBR Green PCR Master Mix, (Qiagen, CA, USA) in CFX96 real time PCR DET SYS (C-1000 thermal cycler, BIO-RAD laboratories India). The real-time RT-PCR experiment was carried out according to the manufacturer's instruction and the following thermal cycling profile was used (40 cycles): 95°C for 15min, 94°C for 15 sec, 59°C for 30 sec and 72°C for 30 sec. Details of the primers used are given in Table 1. The $^{-d}$CT method of relative quantification was used to determine fold change in expression and was obtained as 2^{-d}CT (Livak and Schmitten, 2001).

ELISA

The tissue lysates were quantitatively assayed for SOD (Cayman Chemical, MI, USA), catalase (Cayman Chemical), and GPx (Cayman Chemical), levels as per the manufacturer's instructions.

Statistical Analysis

All data are expressed as mean ± standard error and data were analyzed by two way analysis of variance (ANOVA) followed by Bonferroni's post test using the GraphPad Prism v4.03 software program (San Diego, CA, USA), and the statistically significant differences were considered at $p<0.05$.

RESULTS AND DISCUSSION

In wound, hypoxia causes elevation in HIF-1α which stimulates synthesis of VEGF and SDF-1α by transactivation of respective genes (Milovanova *et al.,* particularly where vasculogenesis is compromised and low grade inflammation persists.

Table 1:
Description of primers used

S.No.	Gene	Primer Sequences	Product size	Annealing Temp.	Accession Number
1	β-actin	F: 5'- TCCTAGCACCATGAAGATCAA G-3' R: 5'- GACTCATCGTACTCCTGCTTG-3'	132	59°C	NC_005111
2	HIF-1α	F: 5'-GGAGCCTTAACCTATCTGTCAC-3' R: 5'-AAGGGAGCCATCATGTTCC-3'	125	59°C	NC_005105
3	SDF-1α	F: 5'-GAGCCAACGTCAAACATCTG-3' R: 5'-GGCTTTGTCCAGGTACTCTTG-3'	137	59°C	AC_000072

Table 2:
Effect of topical application of SP on levels of SOD, catalase and GPx on days 3, 7, 14 and 19 at wound site in diabetic rats by ELISA.

Parameter	Group	Days post-wounding			
		Day 3	Day 7	Day 14	Day 19
SOD (U/mg protein)	Control	12.92±1.08	89.69±21.44	97.96±7.95	132.55±9.95
	Gel	16.63±1.72	177.28±32.47[a]	137.7±14.09	164.61±19.58
	SP	15.24±1.53	169.65±27.20[b]	128.91±17.99	184.33±9.29
Catalase (nmol/min/mg protein)	Control	149.76±26.7	252.98±23.3	367.57±65.3	323.81±27.6
	Gel	266.55±27.25	302.22±29.3	475.13±63.5	386.17±30.9
	SP	203.64±23.6	329.5±24.6	535.18±58.1[b]	418.09±20.7
GPx (nmol/min/mg protein)	Control	41.11±12.2	49.71±8.6	67.83±12.0	88.32±11.9
	Gel	65.85±12.7	74.22±12.3	97.05±8.8	100.56±8.7
	SP	64.51±13.8	79.72±7.8	116.96±15.5[b]	113.18±6.5

Data are expressed as mean ± SE, (n=5). [a or b] $P<0.05$; [a] gel vs. control on the same day; [b] SP vs. control on the same day.

2009). The EPCs in the bone marrow mobilized by wound-induced hypoxia react to chemokine gradients of VEGF and SDF-1α, which result in the homing of these cells to sites of hypoxia where they then participate in the formation of new blood vessels (Gallagher *et al.,* 2007). In the present study, the mRNA expression of HIF-1α was lower in SP-treated wounds throughout the experiment and was significantly lower on day 14 and 19 in comparison to control group (Fig. 1A). However, the expression of SDF-1α was higher in SP-treated wounds during the entire study and significantly increase was observed on day 7 in comparison to control group (Fig. 1B). So, the increased expression of SDF-1α by the application of SP might cause the increased homing of EPC at the wound site supporting increased vasculogenesis and decreased hypoxia at wound site. The significant decreased expression of the HIF-1α on days 14 and 19 further ascertain improved hypoxic conditions in SP-treated wounds. However, in control group, significantly higher expression of HIF-1α on days 14 and 19 post-wounding supported the persistent hypoxic state at wound site. Interestingly, increased HIF-1α expression could not able to increase the SDF-1α expression in control group of present study. Decreased expression of SDF-1α could be due to the impairment in the HIF-1α mediated transactivation of target genes due to decreased association of HIF-1α and its coactivator p300 (Thangarajah *et al.,* 2009). Thus, the decreased association impaired the expressions of SDF-1α and subsequently might decrease the homing of EPC (Gallagher *et al.,* 2007).

The SOD levels were very low in all the three groups on day 3, as compared to later post-wounding days. Afterward, SOD levels was markedly (p<0.01) higher in gel- and SP-treated groups on day 7 (Table 2). The levels of catalase (Table 2) and GPx (Table 2) were higher in SP-treated group from day 7 onwards in comparison to other groups and the significant increase was observed on day 14, as compared to control. Prolonged infiltration of neutrophils, links to the overproduction of reactive oxygen species (ROS), reactive nitrogen species (RNS) and elastases within the wound area with a remarkable cytotoxic and pro-degradative potential (Schonfelder *et al.,* 2005). In this study, significant decreased levels of SOD, catalase and GPx in control group revealed the absence of an appropriate compensatory response from the cell's endogenous antioxidant network, which leads to increased oxidative stress by ROS/RNS. This was might be to the persistence inflammation and hyperglycemia. In our earlier studies on diabetic wounds of rats, we have observed the persistence of inflammation characterized by marked presence of inflammatory cells and increased expression of IL-1β, TNF-α, and MMP-9 in the wounds, which might be responsible for producing oxidative stress at wound site (Kant *et al.,* 2014; Kant *et al.,* 2015). Hyperglycemia adds to the oxidative stress when the production of ROS surpasses the antioxidant capacity (Vincent *et al*., 2004).

In summary, the results of this study revealed that SP has the potential for vasculogenesis at wound site by the up-regulation of SDF-1α gene and cytoprotectant action by decreasing oxidative stress produced by low grade persistent inflammation. This makes it a very good agent for faster wound healing in complicated cases particularly where vasculogenesis is compromised and low grade inflammation persists.

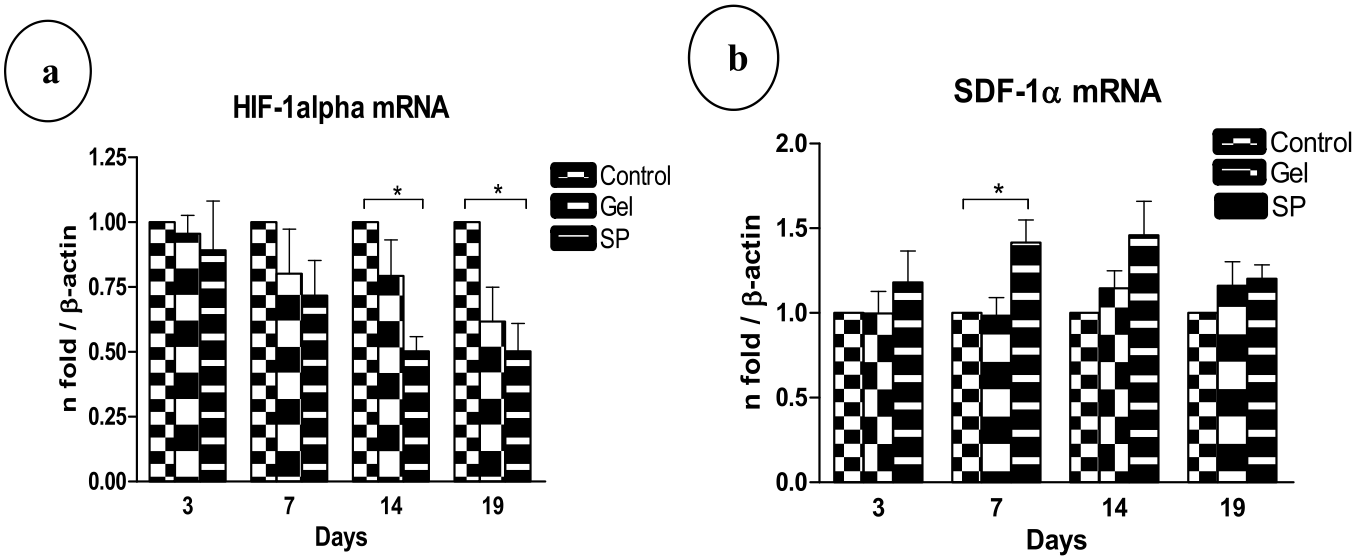

Fig. 1
The mRNA expression of HIF-1α (a) and SDF-1α (b) in healing tissue of control, gel-treated and SP-treated rats on days 3, 7, 14 and 19 post-wounding. The mRNA expressions were normalized by β-actin at each time point and data are expressed as means ± SEM fold change. * p<0.05 vs other group(s) on the same day.

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Research Article

ASSESING THE EFFECT OF GIR COW URINE DISTILLATE ON IMMUNITY AND HEMATO-BIOCHEMICAL PARAMETERS IN MICE

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ABSTRACT

The study was planned to evaluate immunostimulatory effects and hemato-biochemical alterations following 28 days repeated oral administration of cow urine distillate in normal healthy mice. The study was conducted on twenty four (24) male swiss albino mice dividing them in four groups having six mice in each group. Group I served as normal control group given normal saline orally for 28 days. Group II, III and IV served as treatment group given cow urine distillate @ 2 ml/kg, 4 ml/kg and 6 ml/kg, body weight orally for 28 days. At the end of experiment, all the mice were subjected to blood collection; blood and serum sample were analyzed for hematological and biochemical parameters, respectively. Humoral and cell mediated immune response were measured and histopathogical examination of spleen and thymus were carried out. The result obtained clearly indicated that the oral administration of cow urine distillate in normal mice showed immunostimulatory effect by increasing both the antibody titer and cell mediated immune response.

Keywords: Cow urine distillate, immunity, hemato-biochemical alterations, mice

INTRODUCTION

The immune system of an individual is highly susceptible to a number of clinical conditions, cancer, surgery or to the administration of chemical drugs and pollutants in feed. Stress either physical or psychological also causes immune dysfunction. The environmental pollutants such as pesticides, heavy metals and mycotoxins present in various food may alter immunity and suppression of immune system allows opportunistic pathogens to over whelm and cause mortality (Koller, 1979; Chauhan and Chandra, 1997). It has been observed during last few years that desired protection is not achieved against diseases for which animals, various chemical drugs such as aluminium compounds, dextran sulphate and levamisole are used (Quinn, 1990). In Indian ancient literature several herbal preparation have been described, which can be given to animals in order to augment the immune response (Bhargava and Singh, 1981). In this direction earlier some herbal preparations have been studied for their immunomodulatory properties (Chauhan, 1999; Chatterjee, 1994). Panchgavya therapy plays an important role in ayurvedic system of medicine. Panchgavya means the mixture of natural products of cow like milk, curd, ghee, urine and dung. In the ancient literature, it is mentioned that it may increase the resistance of the body. But there seems to be no/few authentic scientific report about the efficacy of panchgavya components. Present study was carried out in mice to investigate the immunostimulatory properties of "Cow urine distillate", a preparation from Gir cow urine.

MATERIALS AND METHODS

Experimental Animals

The study was conducted on 24 healthy male swiss albino mice of 6-8 weeks of age. Mice were procured from Zydus Research Centre (ZRC), Ahmedabad, India. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22 ± 2^0C) and humidity (55 ± 5%) with 12 h light and12 h dark cycle. All the mice were fed with commercially available mice normal pellet diet and deionized water was provided *ad libitum* throughout the course of the experiment. All the mice were kept under acclimatization for 5 days prior to grouping and initiation of experiment. Mice were kept under constant observation during entire period of study. All necessary managemental procedures were adopted to keep the mice free from stress. The protocol of this study was approved by IAEC.

Preparation of cow urine distillate

Three apparently healthy pure Gir cows aged 3-4 years, maintained at Livestock Research Station, College of Veterinary Science & A. H., Anand raised under standard feeding and management conditions, were selected as donor of the urine. Early morning cow urine was collected in sterilized plastic bottles and the samples were brought to the laboratory for preparation of Goumutra ark (Cow urine distillate). It was prepared by boiling cow urine at 100 °C in distillation apparatus, as per the method outlined by Khanuja (2002).

Experimental design

All the mice were randomly divided into four groups

(I, II, III and IV) each containing 6 mice. Animals of all groups were given different treatment for 28 days. Mice of group I were administered normal saline and served as vehicle control group. Group II, III and IV were received Cow urine distillate @ 2 ml, 4 ml and 6 ml per kg body weight respectively, orally for 28 days.

Body weight and feed consumption were recorded at weekly interval. On 29th day of the experiment, blood samples were collected from the retro-orbital plexus with the help of capillary tube before sacrificing the mice. Blood samples were collected in vials containing K_3 ethylene diaminetetra-acetate for hematology (differential leucocyte counts and total leucocyte counts) and in plain vials for serum biochemical estimation (albumin, globulin and total protein) and sheep red blood cells (SRBC) antibody titer by hemagglutination. After sacrificing the mice, spleen and thymus were collected for histopathological examination. Total leukocyte counts (nos/microliter) and differential leukocyte counts (lymphocyte, granulocyte and monocytes) were estimated by hematology autoanalyzer (Mindray; BC – 2800 Vet, China).

Immunization of mice was done using SRBCs. SRBCs were collected in Alsever's solution (composed of 20.5 g of dextrose, 8 g of sodium citrate, 4.2 g of sodium chloride, 0.55 g of citric acid in one liter distilled water), washed in large volumes of sterile 0.9% normal saline thrice and adjusted to a concentration of 5×10^9 cells/ml, were used for immunization. Animals were immunized by injecting 0.2 ml SRBC suspension intraperitoneally 7 days prior to sacrifice (on 21st day of the experiment). Blood was collected from retro-orbital plexus under ether anesthesia on 29th day, and serum was separated to determine the antibody titer by the Hemagglutination test (HA).

Antibody titer

Antibody titer was carried out by diluting the test serum two fold times in 0.15 M phosphate buffer saline (PBS) and aliquoted in "U" bottomed microtiter plates. 1% SRBC suspended in PBS was dispensed in each well and mixed thoroughly. The plates were incubated for 4 h at 37°C and then observed visually for hemagglutination. The highest dilution of the test serum giving hemagglutination was taken as antibody titer.

Assessment of cell-mediated immune response

Cell-mediated immune response was assessed by the method as described by Lagrange *et al.* (1974). All the animals under various groups were immunized by injecting 20 µl of 5×10^9 SRBC/ml subcutaneously into the right foot pad on 19th day of the treatment. Thickness of left foot pad was measured using vernier callipers on 26th day of the treatment. The mice then challenged by injecting 20 µl of 5×10^9 SRBC/ml intradermally on the left hind foot pad (time 0). Foot pad thickness was measured at 24 and 48 h of challenge. The difference in mm was taken as a measure of delayed type hypersensitivity (DTH).

On 29th day of study, all the mice from each group were sacrificed by cervical dislocation and subjected to post mortem examination in the confined disinfected laboratory to determine the presence or absence of gross and histopathological lesions. Post mortem findings were made by systematic approach. Detailed post mortem lesions from all the mice were recorded. For gross (macroscopic) lesions thymus and spleen were collected and examined after opening the body of sacrificed experimental mice. For histopathological examinations, tissues from spleen and thymus were collected in 10% formalin and preserved for processing.

The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections were cut at 6–8 µ thickness with automatic section cutting machine (Leica, Automatic Microtome Machine, Germany), and were stained with Hematoxylin and Eosin (H and E) stains. The H and E stained slides were observed under microscope and lesions were recorded.

Statistical Analysis

All the data have been presented as mean ± SE. Statistical comparisons of the results were made using one way analysis of variance (ANOVA) by using computer software SPSS (Version 20). Significant differences ($p < 0.05$) between different experimental groups were analyzed by Duncan's test.

RESULTS

There was non significant alteration were observed in body weight of treatment groups (II, III and IV) as compared to vehicle control group I. Feed consumption was also found non significant up to third week of experiment but at fourth week of experiment feed consumption was significantly increase in group IV as compared to vehicle control group. Results obtained in relation to hematological assessment are presented in Figure 1. There was significant increase ($p < 0.05$) in total leucocyte count in Cow urine distillate treated group III and group IV as compared to vehicle control group I. The increase in total leucocyte counts was in dose dependent manner. There was significant increase ($p < 0.05$) in lymphocyte count in Cow urine distillate treated group II, group III and group IV as compared to vehicle control Group I in dose dependent manner.

There was non significant difference in serum total protein, serum albumin and globulin level in Cow urine distillate treated groups (II, III and IV) as compared to control group I (Figure 2). There was significant increase ($p < 0.05$) in antibody titer in Cow urine distillate treated group IV as compared to control group I (Figure 3). There was significant increase ($p < 0.05$) in foot pad skin

thickness in Cow urine distillate treated group III and group IV as compared to vehicle control group I at 24 h and 48 h (Figure 4). The increase in skin foot pad thickness is in dose dependent manner. On microscopic examination of spleen and thymus showed normal architecture of spleen and thymus (Plate 1 and Plate 2, respectively).

DISCUSSION

Immunostimulatory effects of Cow urine distillate in this experiment was assessed by estimation of total leucocyte count, lymphocyte count, serum total protein, serum albumin level, humoral and cell mediated immunity.

In present study, there was non-significant difference in body weight between the Cow urine distillate

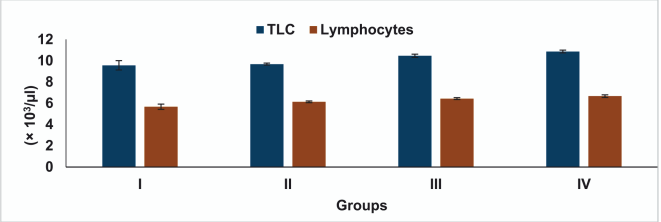

Fig. 1:
Effect of daily oral administration of Cow urine distillate on total leucocytes count (TLC) and lymphocyte count in mice.

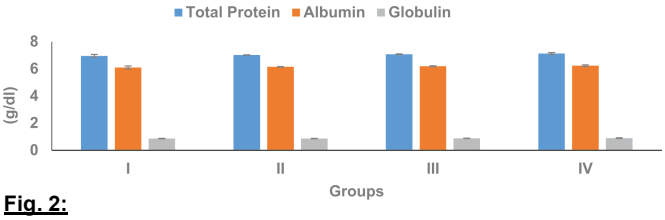

Fig. 2:
Effect of daily oral administration of Cow urine distillate on total protein, serum albumin and globulin level in mice.

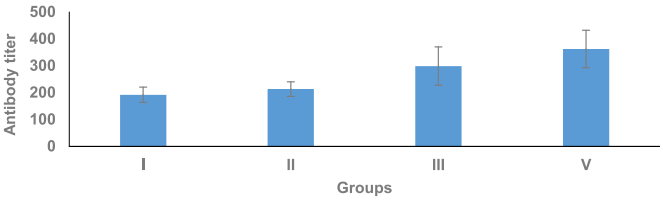

Fig. 3:
Effect of daily oral administration of Cow urine distillate on antibody titer in mice.

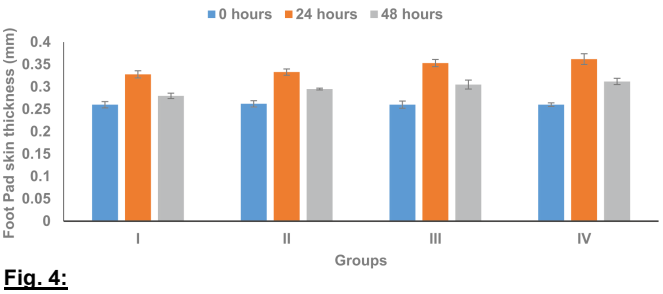

Fig. 4:
Effect of daily oral administration of Cow urine distillate on foot pad skin thickness in mice.

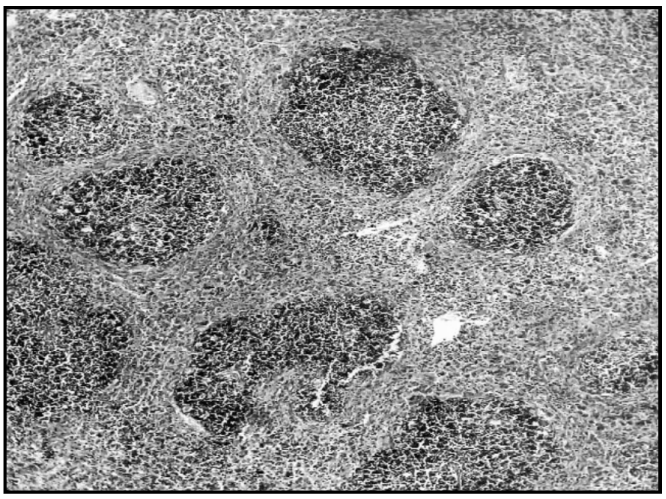

Plate 1:
Section of spleen from Cow urine distillate treated group IV showing clear distinction between the white pulp and red pulp (H & E × 100).

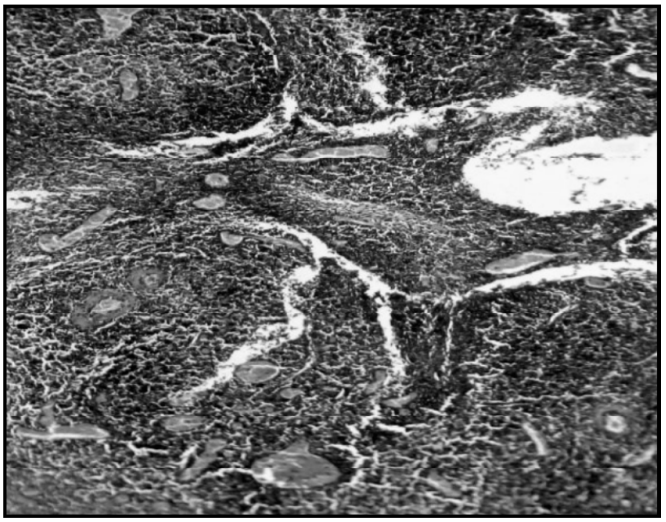

Plate 2:
Section of thymus from Cow urine distillate treated group IV showing dark cellular outer cortex and paler less cellular medulla (H & E × 100).

treated groups as compared to vehicle control group. Similar result also reported by Shukla *et al.* (2013) with non-significant alteration in body weight gain by Cow urine distillate (1 ml and 2 ml per kg) given for 28 days in rats as compared to vehicle control group. However, Chauhan *et al.* (2001) reported that Cow urine distillate (1 ml per 10 mice) found to be improvement in body weight in mice when given for 90 days. At fourth week, there was increase in feed consumption in Cow urine distillate (6 ml/kg) treated group as compared to vehicle control group. This was found similarity with study reported by Garg *et al.* (2005) that the Cow urine (1 ml per bird) with the basal diet increases the feed consumption efficiency and feed conversion ratio in white leghorn layers.

In present study, there was significant increase

($P < 0.05$) in TLC in Cow urine distillate @ 4 ml and 6 ml per kg ($10.467 \pm 0.143 \times 10^3$/µl and $10.867 \pm 0.131 \times 10^3$/µl) treated group as compared to vehicle control group ($9.567 \pm 0.433 \times 10^3$/µl) and lymphocyte count in Cow urine distillate @ 2 ml, 4 ml and 6 ml per kg ($6.133 \pm 0.080 \times 10^3$/µl, $6.433 \pm 0.084 \times 10^3$/µl and $6.667 \pm 0.128 \times 10^3$/µl, respectively) treated group as compared to vehicle control group ($5.667 \pm 0.242 \times 10^3$/µl). The lymphocytes are considered as the main component of the immune mechanism, which is responsible for the main foundation of both the humoral and cell-mediated immunity in the body through B and T cells. Besides, there are lymphocytes in the peripheral blood, which do act against extraneous substance to protect the body non-specifically. Increase in the number of lymphocyte with increase in total leucocyte count showed immunostimulatory effect. Similarly, Verma *et al*. (2011) also found the increase in TLC ($6.8 \pm 1.4 \times 10^3$/µl) in rats by administration of dry cow urine distillate @ 500 mg/kg body weight for 25 days as compared to vehicle control group ($6.3 \pm 2.9 \times 10^3$/µl). Similar results were also found by Sanganal *et al.* (2012) that Cow urine distillate @ 0.05, 0.1, 0.2 and 0.3 ml in a single dose daily for 28 days significantly increase the TLC and lymphocyte count in rats. Additionally, Joshi *et al.* (2012) found increase in TLC by 29.8 % and lymphocyte count by 24.5 % on 60th day of experiment in rabbit. Joshi and Chauhan (2013) and Naseema *et al*. (2014) founded increase level of TLC and lymphocyte in mice treated with cow urine distillate for 180 days and 19 days, respectively.

In present study, there was non-significant increase in the total protein, serum albumin and globulin level in Cow urine distillate given groups as compared to vehicle control group. Similarly, Sanganal *et al.* (2011) found to be non significant increase in total protein level by administration of Cow urine distillate (0.05, 0.1, 0.2 and 0.3 ml) in rats. However, Panicker *et al.* (2012) found to be significant increase in total protein and albumin level in birds administrated Cow urine distillate (1 ml/bird for 90 days). These may be due to anabolic effect of Cow urine distillate on protein metabolism (Panicker *et al.* 2012).

Results obtained in relation to humoral and cell-mediated immune responses are presented in Figures 2 and 3.There was significant increase ($P < 0.05$) in the antibody titer against SRBC in mice given Cow urine distillate @ 6 ml/kg (362.66 ± 69.456) as compared to control group (192 ± 28.622). This increase in antibody titer suggest that Cow urine distillate has immunostimulatory activity. Similarly, Gosavi *et al.* (2011) also found to be increase in antibody titer against SRBC by administration of Cow urine distillate (0.2 ml per rat for 14 days) by 141.33 ± 98.32 as compared to vehicle control (8.33 ± 6.25). Naseema *et al.* (2014) also found similar results in swiss albino mice by administration of Cow urine distillate @ 10.8 ml/kg for 19 days. Additionally, Gupta *et al.* (2004) reported that Cow urine distillate increase the humoral immune response by estimation of immunoglobulin through the sulphite turbidity test.

In present study, there was significant increase in cell mediated immunity against sheep red blood cell in Cow urine distillate (4 ml and 6 ml per kg) given groups (0.353 ± 0.008 mm and 0.362 ± 0.012 mm at 24 h; 0.305 ± 0.010 mm and 0.312 ± 0.007 mm at 48 h) as compared to vehicle control group (0.328 ± 0.008 mm at 24 h and 0.280 ± 0.006 mm at 48 h) at 24 and 48 hours. This was due to increase in T lymphocyte concentration. This increase in foot pad thickness suggest that Cow urine distillate increase the cell mediated immunity. Similar result reported by Verma *et al.* (2011), that the administration of freeze dry Cow urine distillate (500 mg/kg for 25 days) increases the foot pad skin thickness against sheep red blood cells (intradermally) in Cow urine distillate treated group 0.47 ± 0.05 mm (38%) as compared to vehicle control group 0.34 ± 0.03 mm. Naseema *et al.* (2014) also found similar results in swiss albino mice by administration of Cow urine distillate @ 10.8 ml/kg for 19 days. Additionally, Gupta *et al.* (2004) reported increase in skin thickness by delayed type hypersensitivity reaction against 1 % dinitro-chloro benzene in experimental rats and Shakya *et al.* (2013) reported similar result with Cow urine distillate (1 ml/bird/day for 48 days) given group (2.35 ± 0.27) as compared to vehicle control group (2.20 ± 0.31) in chicks. Cell mediated immunity by using T-lymphoblastogenesis were performed using Con-A (Concavallin A) mitogens. Cell mediated immnuty by using T- lymphoblastogenesis was checked by Chauhan *et al*. (2001) in mice and Kumar *et al.* (2005) in chicks. Result showed that T lymphoblastogenesis in Cow urine distillate treated group was significantly higher as compared to vehicle control group. This suggest that Cow urine distillate stimulate the cell mediated immunity so has immunostimulatory activity.

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Research Article

EVALUATION OF SPASMOLYTIC ACTIVITY OF HYDROETHANOLIC EXTRACT OF *ERYHTRINA VARIEGATA* IN ISOLATED RAT ILEUM AND ITS PHYTOCHEMICAL ANALYSIS

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Abstract

The study was carried out to demonstrate the effect of hydroethanolic extract of *Erythrina variegate* leaves (HEVL) on the motility of isolated rat ileum and for phytochemical analysis of HEVL. Qualitative phytochemical analysis of HEVL was done and the smooth muscle activity of HEVL was evaluated by preincubating the tissue with extract for five minutes and then adding standard spasmogenic agents (Acetyl choline and Carbachol), for depicting the spasmolytic effect of HEVL. Phytochemical analysis of HEVL revealed presence of alkaloids, flavonoids, proteins, sterols, saponins and terpenes. The present study also demonstrated the antispasmodic activity of HEVL (100-1200 µg/ml) on isolated rat ileum.

Key words: *Erythrina variegata,* ileum, invitro, rat, phyotchemical analysis.

INTRODUCTION

Erythrina variegata also called *Erythrina indica* is a thorny deciduous tree growing to 60 feet tall. A wide range of chemical compounds have been isolated, mainly alkaloids, flavonoids, triterpenoids, and lectin. The alkaloids extracted from the leaves of *Erythrina variegata* are reported to have anti-inflammatory and analgesic activity. Isoflavonoids isolated from *E. variegata* having antibacterial and anthelmintic activity. *E. variegata* shows several other characteristic pharmacological effects like neuromuscular blockade, smooth muscle relaxant, CNS depressant, and hydrocholeretic (Kumar *et al*., 2010). In view of wide clinical application and unavailability of data to establish the spasmolytic action, the present study was undertaken to evaluate the activity of hydroethanolic extract of *Erythrina variegata* in isolated rat ileum and phytochemical analysis of the extract.

MATERIALS AND METHODS

Plant material and chemicals

The plant material were collected from the GBPUAT, Pantnagar campus and taxonomically identified vide letter no. *CHN/64/2012/Tech-II dated 2.10.2012*, Office of Scientist 'F', Central National Herbarium, Botanical Survey of India, Botanical garden, Howrah 711-103 as *Erythrina variegata* (L.). The fresh leaves of the plant were used in the investigation.

Ammonia, benedict's reagent, benzene, chloroform, conc. sulphuric acid, copper sulphate, ethanol, ferric chloride, hydrochloric acid, iodine, magnesium turnings, methanol, potassium iodide, sodium bicarbonate and sodium hydroxide were purchased from Hi-media.

Acetylcholine chloride (Sigma-Aldrich), atropine sulfate (Hi-media), epinephrine hydrochloride (Sigma), prazosin (Sigma), propranolol (Sigma), calcium chloride (S. D. Fine-Chem Ltd.) and potassium chloride (Hi-media).

Preparation of the hydroethanolic extract

The plantwas chopped into small pieces. These were shade dried followed by drying in incubator at 35-40°C for 3-4 days to remove excess moisture. The dried plant was grinded in mixer to obtain a fine homogeneous powder. The powder of *Erythrina variegata* plant was dark olive in colour. The powder was stored in sealed plastic container in a dry place at room temperature to prepare 50% hydroethanolic extract was prepared by extraction of fine powder in 50% ethanol. To prepare cold extracts (Singh, 2008).

Analysis of extract for presence of phytochemical groups

Qualitative chemical analysis of hydroethanolic extract of Erythrina *variegata* was done to detect major phytochemical groups viz., alkaloids, anthraquinones, flavonoids, saponins, tannins, sterols, reducing sugars, glycosides, resins, triterpenes and proteins by standard methods (Harborne, 1973; Sofawara, 1982).

Tissue mounting on organ bath

To evaluate the effect of HEVL on smooth muscle activity, isolated rat ileum was used for the study. The ileum was collected from the control group rats of the *in-vivo* experiment. The animal was off-fed overnight before sacrifice. After anaesthetizing with ether, ileum was dissected out, free from fat and connective tissue and immediately transferred into cold (4°C) aerated Tyrodes solution and then mounted on isolated organ bath.

A 10-15 mm long piece of rat ileum was mounted with one end knot to the oxygen tube and other end to a S-shaped key hung on the transducer by means of a thread and then immersed in organ tube filled with 40 ml of Tyrodes

solution (37°C). The temperature of the organ bath was thermostatically controlled and the tissue was continuously aerated by an aerator attached to the oxygen tube. After mounting, the tissue was kept for an equilibration period of 90 minutes. During this period, the bathing fluid was changed every 15 minutes. After equilibration period, the drugs were added as per the experimental protocol. The response was recorded on student's physiograph (BSL Pro 3.7) and analysed by comparing the deviation from baseline.

Experimental protocol

The effect of plant extract on smooth muscle activity of rat ileum was observed in the following manner.

Effect of acetylcholine on HEVL sensitized rat ileum

Acetylcholine (10^{-5}M) was added to organ tube, allowed to act for one minute and then washed, the same procedure is repeated 5 times and standard contraction produced by acetyl choline is recorded (Fig. 1a). After 5 minutes, atropine sulfate (10^{-4}M) was added and allowed to act for one minute,this was followed by addition of acetylcholine (10^{-5}M), preincubating the tissue with atropine sulphate leads to complete attenuation of contractile response showed by acetyl choline. The similar protocol was followed for HEVL (100-1200µg/ml) where atropine sulfate was replaced by extract.

Effect of carbachol on HEVL sensitized rat ileum

Carbachol (10^{-5}M) was added to organ tube, allowed to act for one minute and then washed, five replicates of the contractile response shown by carbachol was done and standard contractile response shown by carbachol is recorded (Fig. 1b). After 5 minutes, atropine sulfate (10^{-4}M) was added and allowed to act for one minute, this was followed by addition of carbachol (10^{-5}M), to observe the effect shown by carbachol in presence of atropine. Atropine completely abolished the contractile response showed by carbachol.The similar protocol was followed for HEVL (100-1000µg/ml) where atropine sulfate was replaced by extract.

RESULTS AND DISCUSSION

The recovery of the hydroethanolic extract was found to be 8 %. The phytochemical analysis revealed the presence of alkaloids, flavonoids, proteins, sterols, saponins and terpenes. Anthraquinones, glycosides, reducing sugars resins and tannins were not found (Table 1).

The results of phytochemical analysis are in agreement with the findings of Devi and Manoharan (2011), who reported the presence of alkaloids, steroids, phenol, saponin and coumarin. However, they did not found flavonoids in their investigation. The presence of flavonoids in our analysis might be due to variation in the source of plant or due to difference of solvent used for extraction.

Presensitization of rat ileum with HEVL (100-1200 µg/ml) for five minutes attenuated the contractile response produced by acetylcholine indicating spasmolytic property of the extract. The results are shown in *Figure2 and 3*as percent contraction inhibition produced by HEVLpresensitization on acetyl choline induced contraction.

Table 1:
Phytochemical analysis of hydroethanolic extract of *Erythrina variegata*(HEVL)

Phytochemicals	Method	Observation
Alkaloids	Wagner	+
Anthraquinones	—	-
Flavonoids	—	+
Glycosides	—	-
Proteins	Biuret test	+
Reducing sugars	—	-
Resins	—	-
Sterols	Salkowski reaction	+
Saponins	—	+
Tannins	Ferric chloride test	-
Terpenes	—	+

Presensitization of rat ileum with HEVL (100-1000 µg/ml) for five minutes attenuated the contractile response produced by carbachol indicating spasmolytic property of the extract. The results are shown in *Figure 4* as contraction inhibition produced by HEVL presensitization on carbachol induced contraction.

The present study demonstrated the antispasmodic activity of hydroethanolic extract of *Erythrina variegata* on isolated rat ileum. The activity of spasmolytic compounds is determined by their ability to alter the tone of smooth muscles. In order to check the antispasmodic activity in our study, the tissue was preincubated with the extract for five minute and then the standard spasmogenic agent were added. A similar method of preincubating the tissue with extract was also followed by Channa (2001).

The action of acetylcholine on GIT is mediated through muscarinic receptors. Since, the extract attenuated the contractile response produced by acetylcholine and carbachol on rat ileum; it is likely that HEVL possessed antimuscarinic properties like atropine. Antimuscarinic property might be due to presence of alkaloids in the extract as evident from phytochemical analysis conducted in this investigation. Other possible mechanism could be inhibition of calcium influx. Intestinal smooth muscles showed spontaneous rhythmic movements regulated by periodic depolarization and repolarization. At the height of depolarization, there is a rapid influx of Ca^{2+} via voltage-operated calcium channels. The inhibition of spontaneous activity of smooth muscle by HEVL could be either due to interference in the depolarization process or with the calcium influx through voltage-operated channels. The findings on smooth muscle activity observed in this study are in accordance with the findings of Ghosal *et al.* (1972), who also reported spasmolytic activity of alkaloids obtained from bark of

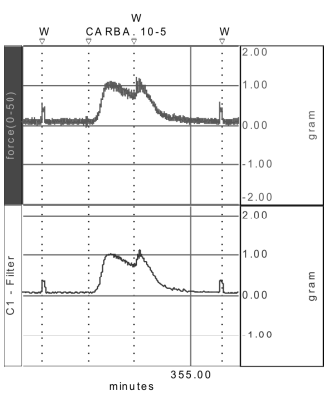

Fig. 1a

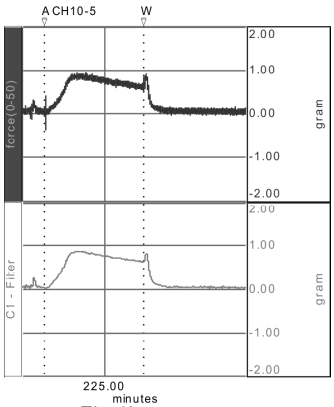

Fig.1b

Fig.1:
1a. Contraction produced by carbachol 10^{-5} M and Fig. 1b. Contraction produced by Ach 10^{-5} M

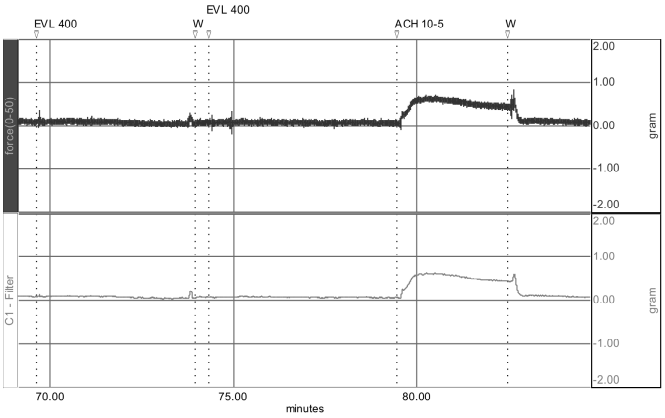

Fig.2:
Spasmolytic effect of HEVL @ 400 µg/ml on Ach induced contraction

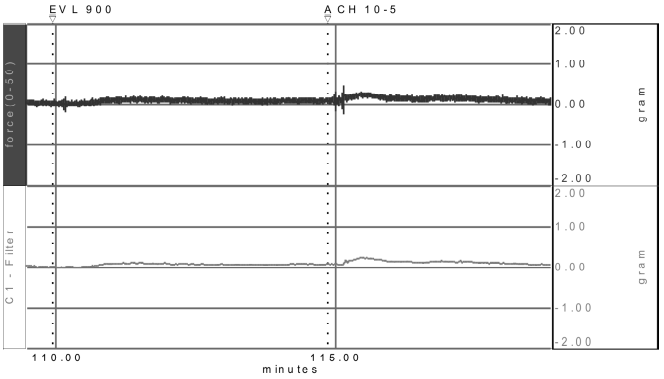

Fig.3:
Spasmolytic effect of HEVL @ 900 µg/ml on Ach induced contraction

Erythrina variegata in isolated rat uterus.

It is concluded from the present investigation that hydroethanolic extract of *Erythrina variegate* contains alkaloids, flavonoids, proteins, sterols, saponins and terpenes. have spasmolytic activity on smooth muscle activity of rat ileum

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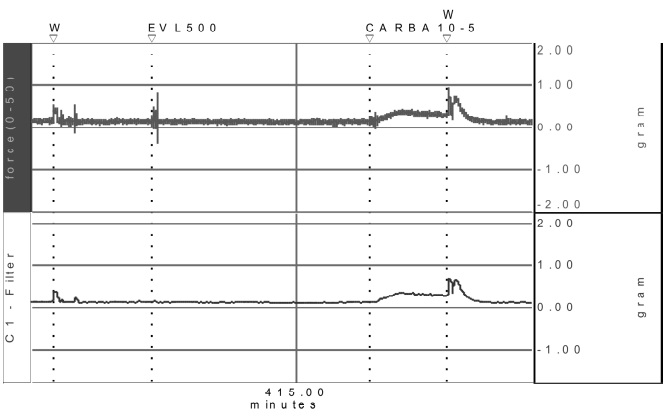

Fig.4:
Spasmolytic effect of HEVL @ 400 µg/ml on Carbachol induced contraction

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Research Article

PHARMACOKINETICS AND DOSAGE REGIMEN OF CEFQUINOME IN HEALTHY FEMALE GOATS FOLLOWING INTRAVENOUS ADMINISTRATION

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ABSTRACT

Pharmacokinetics and dosage regimen of cefquinome @ 2mgkg^{-1}BW was studied in healthy female goats (n=4) following its intravenous administration. Cefquinome concentration in plasma was estimated using HPLC. Plasma disposition kinetics followed three compartment model after intravenous injection. The drug was detected upto 24hr in healthy goats. Following intravenous administration, the main pharmacokinetic parameters (mean ± SEM) were V_d area 0.41± 0.04 LKg^{-1} , AUC 43.57± 1.31 ugml^{-1}h , $t_{1/2\alpha1}$ 0.59 ± 0.04h , $t_{1/2\alpha}$ 4.00 ± 0.59 h , $t_{1/2\beta}$ 6.21± 0.51 h , Cl_B 0.04± 0.001LKg^{-1}h^{-1} and MRT 7.06 ± 0.19 h. For calculating the dosage regimen %T>MIC was observed to be above 90% in healthy goats nevertheless this prediction is not based on the PD parameters from pathogens of goats origin. The integration of pharmacokinetic and pharmacodynamic parameters suggested that cefquinome @ 2mg/kg single i.v. administration is sufficient to maintain desired therapeutic levels in goats for bacteria with MIC≤0.39µgml^{-1}.

Keywords: Pharmacokinetics, goats, cefquinome, pharmacodynamics.

INTRODUCTION

Cefquinome , a fourth generation cephalosporin with broad spectrum of activity against gram-positive and gram-negative bacteria developed exclusively for veterinary use including food animals , is an aminothiazolyl cephalosporin denoted as (1-[(6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)-(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0-oct-2-en-3-yl]methyl]-5,6,7,8-tetra - hydroquinolinium inner salt) (Guerin-Faublee *et al.,* 2003). The aminothiazolyl cephalosporins possess favorable chemotherapeutic properties and are associated with lesser adverse effects (Caprile 1988). The cefquinome molecule differs from third-generation cephalosporins by a quaternary ammonium side chain attached to the C-3 position of the β-lactam nucleus. The chemical modifications of the basic cephalosporin structure provide zwitter ionic property to cefquinome which can facilitate rapid penetration across biological membranes including the porins of the bacterial cell wall, thus enhancing bioavailability and improving the spectrum of antimicrobial activity compared with the second and third generation cephalosporins (Thomas *et al.,* 2006). Also as a result of their molecular structure, cefquinome is stable against chromosomally and plasmid-encoded β-lactamases that are produced by a majority of clinically important bacteria (Limbert *et al* 1991). It is indicated in the treatment of respiratory tract diseases, acute mastitis and foot rot in cattle, calf septicemia, foal septicemia and respiratory tract diseases in horses (CVMP 2003).

Pharmacokinetics describe the concentration time profile of the any agent including antimicrobials in serum / plasma or any other biological fluids which can be affected by many factors like formulation , administration route, the species , the dose and the disease (Giguere, 2007).The pharmacokinetic studies of cefquinome have been conducted in other species but as there is paucity of data on the pharmacokinetic studies of cefquinome in female goats till date so the present study was conducted to investigate the disposition of cefquinome after single i.v. administration in healthy female goats and to determine the appropriate dosage regimen of cefquinome in these animals.

MATERIALS AND METHODS

The experiment was performed on the locally procured adult female goats of approximately 2-3 years of age. All goats were healthy and not treated with antibiotics for last one month before the beginning of trial. The goats were acclimatized for 2 weeks in the faculty animal shed prior to the commencement of experiments. During this period all animals were subjected to regular clinical examination and treated with anthelmintics for deworming. The animals were maintained on green pasture and concentrate mixture, and water was provided ad libitum. The experimental protocol followed the ethical guidelines on the proper care and use of animals.

Four female goats of weight 20 ± 4 kgs were taken and to them cefquinome sulfate powder with solvent for injection (Intas Pharmaceuticals Ltd ,Ahmedabad, India) was administered intravenously into the jugular vein at single dose of 2 mg/kg. The dosage level of cefquinome employed in the present study was based on a pilot and previous studies (Uney *et al.,* 2011).

Blood samples were collected by jugular

venipuncture in heparinised sterile test tubes at time periods : 0 , 1 , 2.5 , 5 , 10 , 20 , 30 , 60 min and 2 , 4 , 6 , 8 , 12 and 24 h after drug administration.

Assay procedure

Plasma concentrations of cefquinome were determined using high performance liquid chromatography (HPLC) as per the method of Uney *et al* (2011). The HPLC (Perkin Elmer) consisted of a single pump (Perkin Elmer series 200), a degasser (Perkin Elmer series 200) and an autosampler injector with 200 µl loop (Perkin Elmer series 200).The detection was performed using an UV/VIS detector (Perkin Elmer 200 series) set at 268 nm. The reverse-phase chromatography was performed with an analytical C_{18} column (Merck®, Particle size 5µ, 4.6 × 150 mm, Waters, USA). The optimized method used binary-gradient mobile phase with water containing 0.1% TFA as mobile phase A and ACN as mobile phase B. The time program of the gradient is listed in the following Table 1.

The retention time of cefquinome was about 6.1 min with a total run time of 15 min. The linearity of the method was evaluated by a calibration curve in the range of 0.05 to 50 µg.ml-cefquinome (Fig 1). The LOQ of the method was found to be 0.04 µg.ml^{-1} for cefquinome in goat plasma with acceptable accuracy and precision (<15% for each criterion). The LOD was determined to be 0.02 µg.ml^{-1} based on a signal-to-noise ratio of 3:1.

Sample processing

Plasma samples (200 µl) were added to 2 ml microcentrifuge tubes and to all samples 400 µl of MeOH was added and mixed for 10 seconds (s) and the samples were centrifuged at 4000 × *g* for 10 min. After centrifugation, 300 µl of clear supernatant was pipetted into a fresh vial, 150 µl of water was added and mixed for 10 s and the mixed clear supernatant (200 µl) was pipetted into an autosampler vial. The extracted cefquinome sample (50 µl) was injected into the HPLC system.

Statistical Analysis

The plasma concentrations and pharmacokinetics parameters are expressed as mean ± SE. The differences between two means based on individual observation were determined by student's t-test and level of significance was determined at 1 & 5% as described by Snedecor & Cochran, 1967**.**

Quantification

The regression formula obtained from the calibration curves was used to quantify the concentration of cefquinome in plasma by substituting respective analyte area.

$$y = ax \pm b$$

where,

y= analyte area; *b*= *y* intercept; *a*= slope of the calibration curve ; *x*= concentration (µg ml^{-1})

The regression equation obtained to quantify the concentration of cefquinome in plasma was

$$y = 21474x + 259.17$$

RESULTS

No local or systemic reaction to cefquinome occurred after intravenous administration Pharmacokinetic studies were conducted in goats following single intravenous administration of cefquinome @ 2mg kg^{-1} BW. The various kinetic determinants computed on the basis of plasma Cefquinome concentrations at different time intervals were determined. Semi-logarithmic plot of mean plasma concentrations versus time showed a triphasic curve and thus it fitted to three compartmental open model. The mean plasma concentrations of cefquinome after single dose i.v administration are depicted in Table 2. The disposition curve for cefquinome in healthy goats following single i.v. administration is depicted in Fig 2. Various pharmacokinetic parameters after single dose i.v administration are presented in Table 3 and Table 4. As Cefquinome is a beta lactam antibiotic which usually possess time dependent killing (Thomas *et al*., 2006) so for seeing the efficacy of the given dose of the antibiotic % T > MIC is the best pharmacokinetic / pharmacodynamic factor to describe the efficacy of bacterial killing as it describes the length of the time during which drug

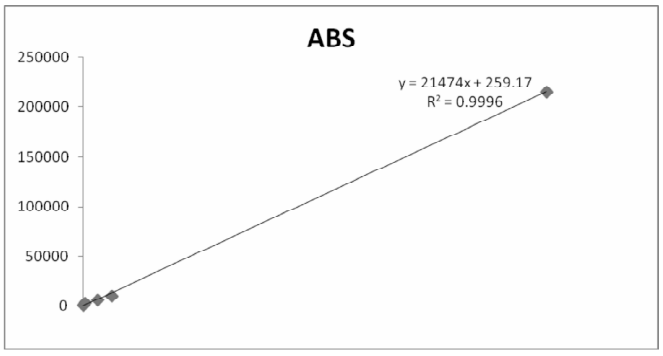

Fig. 1.
Standard curve of different concentrations (µg ml^{-1}) of cefquinome in plasma and their respective areas (µm^2)

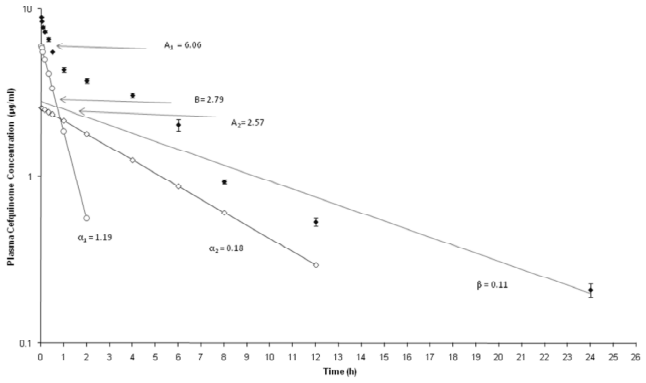

Fig. 2.
A semi logarithmic plot of plasma levels of cefquinome after its single i.v administration at the rate of 2 mg/ kg body weight in healthy goats showing three compartment open model.

concentrations remain above MIC90 value (Craig, 1995; Turnidge, 1998) .Table 5 shows the % T > MIC values of Cefquinome @ 2mg / kg in healthy goats for the bacteria of MIC between 0.035 to 0.39 (Dinakaran *et al*., 2013).

DISCUSSION

The dosage level of cefquinome employed in the present study was based on a pilot study and comparable to previous study in sheep (Uney *et al.,* 2011). The study revealed that Plasma concentration- vs- time profile after intravenous route best fitted three compartment open model in healthy female goats. In healthy animals, the drug appears to be distributed most rapidly into highly vascularised central compartment, less rapidly into shallow tissue compartment and very slowly into deep tissue compartment thus following three compartment open model. The three compartment model has been observed in ceftriaxone in buffalo calves (Dardi *et al*., 2005) and ceftazidime in cows (Rule *et al*., 1996). At 1 min the peak plasma concentration in healthy female goats was 8.83±0.01µg/ml and the drug was detected upto 24 hrs. The distribution half life I ($t_{1/2\alpha1}$) from central to peripheral compartment I was 0.59±0.04 h and from central to peripheral compartment II ($t_{1/2\alpha2}$) was 4.00 ±0.59 h indicating transfer of drug into shallow compartment and deep tissue compartments involving two first order processes. The values of AUC and AUMC were 43.57 ±1.31 µg.ml^{-1}.h and 307.45±5.83µg.ml.h^2 in healthy goats , respectively.

The volume of distribution $V_{d(B)}$ in present study was found 0.74±0.08 Lkg^{-1} indicating that the drug is extensively distributed to various tissues and body fluids .Also low values of $V_{d(ss)}$ (0.32±0.01 Lkg^{-1}) were observed in present study.

Further, T/P ratio of 1.37± 0.18 in healthy animals indicated that the penetration of the drug was more. Total

Table 1:

HPLC mobile-phase gradient conditions for analysis of cefquinome.

Time (min)	Flow rate (ml.min^{-1})	% A	% B
0	0.9	90	10
7	0.9	50	50
3	0.9	50	50
1	0.9	90	10
4	0.9	90	10

A -mobile phase A (0.1% TFA in water), B - mobile phase B (ACN).

Table 2:

Plasma concentration (g/ml) of cefquinome following its single intravenous injection (2mg/kg) in healthy goats.

Time after cefquinome administration	Mean ± S.E
1 min	8.83 ± 0.01
2.5min	8.36 ± 0.03
5 min	7.74 ± 0.11
10 min	7.28 ± 0.08
20min	6.58 ± 0.18
30 min	5.49 ± 0.04
60 min	4.33 ± 0.12
2 hr	3.69 ± 0.09
4 hr	3.03 ± 0.10
6 hr	2.02 ± 0.15
8 hr	0.92 ± 0.03
12 hr	0.53 ± 0.02
24 hr	0.20 ± 0.01

Table 3:

Distribution kinetics parameters of cefquinome in healthy goats following single i.v administration (2mg/kg).

Parameter	Unit	Mean ±S.E
A_1	ugml^{-1}	6.06± 0.48
α_1	h^{-1}	1.19 ± 0.08
A_2	ugml^{-1}	2.57 ± 0.43
α_2	h^{-1}	0.18 ± 0.03
$t_{1/2}\alpha_1$	h	0.59 ± 0.04
$t_{1/2}\alpha$	h	4.00 ± 0.59
K_{31}	h^{-1}	0.64 ± 0.03
K_{13}	h^{-1}	0.41 ± 0.07
K_{13}/K_{31}	ratio	0.65 ± 0.11
K_{21}	h^{-1}	0.14±0.004
K_{12}	h^{-1}	0.02 ± 0.02
K_{12}/K_{21}	ratio	0.18 ± 0.13
AUC	ugml^{-1} h	43.57 ± 1.31
AUMC	ugml^{-1} h^2	307.45 ± 5.83
$V_{d(area)}$	L kg^{-1}	0.41 ± 0.04
$V_{d(B)}$	L kg^{-1}	0.74 ± 0.08
$V_{d\ (ss)}$	L kg-^{1}	0.32 ± 0.01
f_c	Ratio	0.42 ± 0.02
T/P	Ratio	1.37± 0.18

• $t_{½\alpha1}$, $t_{½\alpha2}$ - distribution I and distribution II half lives, respectively, α_1, α_2 - distribution I, distribution II constants respectively, K_{21} - the rate of transfer of drug from peripheral (tissue) to central (blood) compartment, K_{12} - the rate of transfer of drug from central to peripheral compartment, K_{13} and K_{31} - the transfer rate constants of drugs from central to peripheral compartment II and vice versa, V_d - the apparent volume of distribution, $V_{d(area)}$ - based on the total area under the plasma drug-concentration versus time curve, $V_{d(B)}$ - based on zero-time plasma concentration intercept of the least square regression line of elimination phase,$V_{d(SS)}$ - based on average steady state plasma level, f_c - the fraction of administered dose present in the central compartment, AUC - Area under curve, AUMC - Area under moment curve, T/P - ratio of drug concentration between peripheral and central compartments

Table 4 :

Elimination kinetics parameters of cefquinome in healthy goats following single i.v administration (2mg/kg).

Parameter	Unit	Mean ±S.E
C_p^0	ugml^{-1}	11.43±0.33
B	ugml^{-1}	2.79±0.28
β	h^{-1}	0.11±0.008
$t_{1/2\beta}$	h	6.21±0.51
K_{el}	h^{-1}	0.26±0.004
$t_{1/2}K_{el}$	h	2.64±0.05
Cl_B	L Kg^{-1} h^{-1}	0.04±0.001
V_C	L Kg^{-1}	0.17±0.005
t_d	H	14.78±1.21
MRT	h	7.06±0.19

• C_p°- the expected plasma drug concentration at zero time, β - slope of terminal phase of elimination, $t½_\beta$ - elimination half life, K_{el}- elimination rate constant from central compartment, Cl_B- the total body clearance of drug, Vc- the apparent volume of central compartment, t_d- duration of pharmacological effect, MRT- Mean residential time.

Table 5 :
%T > MIC values at different time intervals of cefquinome in healthy female goats @ 2mg / kg single i.v administration.

MIC*(µg / ml)	Dosage interval(h) 24 h	Dosage interval(h) 12 h	Dosage interval(h) 8h
0.035	184.93±11.3	369.86±22.6	554.80±33.92
0.07	158.89±9.13	317.78±8.26	476.67±27.39
0.15	130.25±6.74	260.51±13.4	390.77±20.23
0.39	94.36±3.78	188.72±7.56	283.08±11.34

*T > MIC has been calculated for MICs of range 0.035 to 0.39 µg/mL on the basis of reported MIC of 0.035 ?g / ml for for *Pasteruella multocida* , 0.07 ?g / ml for *E.coli* (Dinakaran *et al*., 2013) and 0.39 ?g / ml for *Streptococcus agalactiae* (Wallmann *et al*., 2006; Limbert *et al*., 1991).

body clearance of cefquinome, which represents the sum of metabolic and excretory process was 0.04±0.001 $L.kg^{-1}.h^{-1}$ in healthy goats The elimination rate constant (β) and elimination half life ($t_{1/2\beta}$) in the present study were 0.11±0.008 h^{-1} and 6.21±0.51 h in healthy animals, respectively.

Dosage regimen and route of administration of an antimicrobial agent are most important factors that determine the drug efficacy and the pharmacodynamic parameters integrate both pharmacokinetic and MIC data. For calculating the dosage regimens for the β-lactam antibiotics these pharmacodynamics relationships are necessarily considered. Since β-lactam antibiotics such as penicillins, potentiated-aminopenicillins and cephalosporins are slowly bactericidal, their concentration ought to be kept above the MIC throughout the dosing interval (long T > MIC) for optimal bactericidal effect (Turnidge 1999). The in vitro efficacy of cefquinome against a wide range of gram-negative and gram-positive bacterial pathogens has been demonstrated by various workers who reported MIC_{90s} (0.035–0.39 µg/mL) for *Escherichia coli, Pasteruella multocida and Streptococcus agalactiae* (Dinakaran *et al*., 2013; Thomas *et al.,* 2006; Wallmann *et al*., 2006) As the minimum inhibitory concentrations of cefquinome for bacterial isolates from goats have not been determined so considering the above cited report, the % T > MIC was calculated for MICs of 0.035, 0.07, 0.15 and 0.39 µg/ml. It has been observed that in both healthy female goats cefquinome at a dose rate of 2 mg/kg body weight i.v. at 24 h interval is sufficient to maintain T > MIC above 90% for bacteria with MIC values d" 0.39 µg/mL. This dosage regimen meets pharmacokinetic–pharmacodynamic criteria predicting a successful therapy for susceptible bacteria with MIC d" 0.39 µg/ml . The calculated dosage regimen is hence suggested for potential clinical testing of cefquinome in goats against susceptible organism.

From the present study it can be inferred that the cefquinome @ 2mg/kg body weight i.v. is sufficient to maintain %T>MIC above 90 % in healthy female goats for bacteria with MICd" 0.39µg/ml. This dosage regimen meets pharmacokinetic–pharmacodynamic criteria predicting a successful therapy for susceptible bacteria with MIC d" 0.39 g/mL, nevertheless this prediction is not based on the PD parameters from the pathogens of goat origin. The calculated dosage regimen is hence suggested for potential clinical testing of cefquinome in goats against susceptible micro-organisms.

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Research Article

EXPRESSION PROFILING OF HSP70 IN LIVER TISSUE FOLLOWING SUBACUTE EXPOSURE TO ETHION IN BROILERS

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ABSTRACT

The present study was conducted to evaluate the toxic effect of sub acute exposure of ethion in broiler in terms of expression of heat shock protein 70 (HSP70). The study was conducted in 24 broiler chicks divided equally and randomly in four groups having six in each group. Group I served as control, whereas, group II, III and IV was exposed to ethion for 30 days at different dose levels i.e. 2.5, 5.0 and 7.5 mg/kg respectively. Birds were sacrificed after 30 days and liver tissue was collected from each group. HSP70 expression was analysed by running the RT-PCR and the expression of HSP70 was recorded in form of fold change. In ethion exposed groups, there was increased fold change i.e. 2.8, 4.6 and 7.8 in groups II, III and IV respectively. The increased expression of HSP70 indicates the ethion on sub acute exposure causes the alteration in normal physiology of the broilers because HSP70 also related with the protein folding and unfolding and thus toxicity may result into disturbed meat and egg quality.

Key words : Ethion, heat shock protein, broiler etc.

INTRODUCTION

Recent data from the Asian countries indicates that every year there are three million cases of severe poisoning and 2,20,000 deaths (Gunnell and Eddleston, 2003 and Buckley *et al.,*2004). Organophosphorus agents cause a sequential triphasic illness in man. Mostly the earliest cholinergic phase may only be observed. This cholinergic phase progresses to the intermediate syndrome in around 20% of subjects. Both the first acute and the second intermediate syndrome are attributed to the high risk of mortality and subjects are best managed in an intensive care unit unless the poisoning has been very mild. The final and last phase of triphasic illness is organophosphate induced delayed polyneuropathy, which does not carry the risk of death, sets in 7–21 days after exposure to an organophosphorus agent and may not be preceded by either the cholinergic phase or the intermediate syndrome (Karalliedde and Senanayake, 1989). Heat stress is one of the most challenging environmental conditions affecting the poultry industry globally. In comparison with the other animals , broiler chickens are more sensitive to high ambient temperatures as they do not possess the sweat glands and they are having high body temperature around 106˚c and rapid biotransformation mechanism. In addition to it, fast-growing lean broilers generate more heat than their free-living counterparts living in the wild (Geraert *et al.,* 1993). The heat shock response (HSR) is one of the prime adaptive stress responses of the cell, restoring cellular homeostasis following exposure to proteotoxic stress, including cold, heat shock, oxidative stress, toxins, chemicals, pathogen, etc (Pockley and Multhoff, 2008; Velichko *et al*., 2013 and Meijering, 2015). Therefore the present study was designed to observe the level of expression in terms of fold change and delta delta Ct value after sub acute exposure to ethion.

MATERIALS AND METHODS

The study was conducted in broiler chicks of either sex randomly divided into 4 different groups comprising of 6 birds each. At the age of 0 day, 24 chicks were procured from CARI and these birds were acclimatized for two weeks in the experimental animal shed of division under standard managemental conditions. Standard poultry feed and water were provided *ad libitum* and kept under constant observation throughout study.

Table 1: *In vivo 30* days sub-acute toxicity study

Sl.	Groups	Dose level	Duration of administration	Route of administration
1	I	Control	30 days	Oral
2	II	2.5 mg/kg	30 days	Oral
3	III	5.0 mg/kg	30 days	Oral
4	IV	7.5 mg/kg	30 days	oral

Expression profiling of HSP 70 by Real Time PCR

Isolation of RNA

After washed in ice-cold physiological saline, approximately 0.5 g of each tissue stored at -70°C was ground in liquid nitrogen with a pestle and mortar. Total RNA was isolated from the ground tissue using trizol. Each RNA pellet was resuspended in 50 μl of RNase-free water and stored below -70°C until used. Total RNA purity was determined by calculating the ratio of the absorbance

readings at 260 nm and 280 nm.

Primer used

The *hsp70 mRNA* primers used were as follows: 52 -AGCGTAACAC CACCA TTCC-32 (forward) and 52 -TGGCTCCCAC CCTAT CTC-32 (reverse). *GAPDH mRNA* was detected using the frequently described oligonucleotide that are specific for broiler *GAPDH mRNA* sequences .The primers used were as follows: 52 -TGAAAGTCGG AGTCA ACGGA T-32 (forward), and 52 -ACGCTCCTGG AAGAT AGTGA T-32 (reverse).

Thermal cyclic conditions

The thermal profile for SYBR Green I-based one step real-time RT-PCR consisted of 50 min reverse transcription at 42°C, and one 3 min cycle of Taq DNA polymerase activation at 95°C, followed by 40 cycles of PCR at 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C for 30 s (extension). A single melt curve revealed amplification of desired product as depicted in figure 1. Only a single melt curve is a proof that our HSP 70 the target gene was amplified, not the others. The result was analysed by using delta delta C_t value which was drawn from the melt curve analysis.

RESULTS AND DISCUSSION

The expression profiling of HSP 70 gene was done with the real time PCR. There was a significant difference in delta delta C_t value in ethion treated groups II, III and IV. There were 2.8, 4.6 and 7.8 fold changes in expression profile of HSP 70 in group II, III and IV respectively (Figure 2 and 3). HSPs may play important roles in protein regulation by having a role in assembly and disassembly, folding and unfolding (Mayer and Bukau, 2004); protein translocation (Ryan and Pfanner, 2001), and the refolding of damaged proteins (Glover and Lindquist, 1998). Among most of the expressed heat shock proteins (HSPs), those with a molecular weight of approximately 70 kDa appear to be most closely associated with heat tolerance (Craig and Gross, 1991 and King *et al.,* 2002). It is evident by previous studies that the stress protein plays an important role in cellular defence mechanism. These proteins including HSP buffer cells by maintaining the protein in right conformation. Heat Shock Proteins (HSPs) are detected in all cells, prokaryotic and eukaryotic. In vivo and in vitro studies have shown that various stressors transiently show the up regulation of the heat shock proteins as an befitting tool against the harmful insults. The expression of HSP depends on the severity of the agent such as heat, pesticides and heavy metals etc. Ethion exposure in broiler leads to increase in fold change that indicates the positive expression of HSP 70. This indicates the ethion exposure for subacute exposure in broilers may lead to over expression thus leads to misfolding and alteration in protein formation. Thus the meat yield and egg production may also decrease by

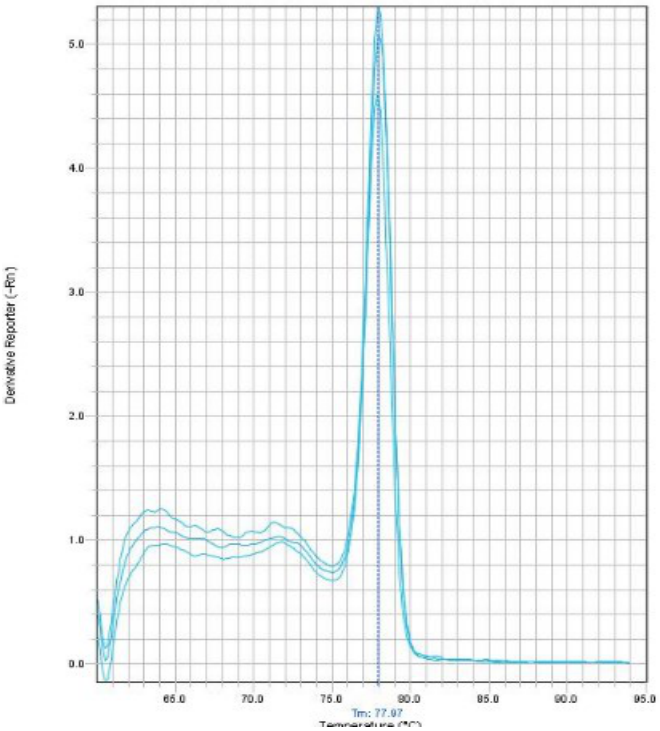

Fig 1:
Melt curve of HSP70 following daily oral administration of ethion for 30 days in broiler chicks (Mean ± S.E., n=6).

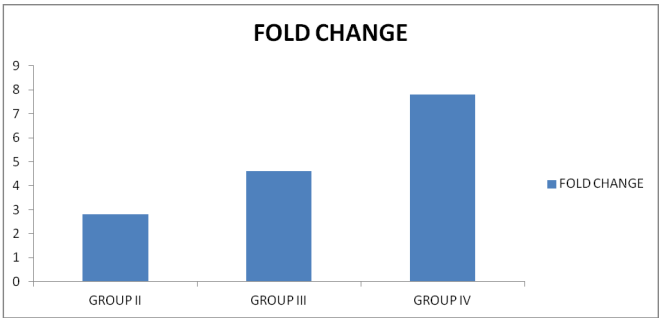

Fig 2:
Effect on HSP 70 expression in terms of fold change following daily oral administration of ethion for 30 days in broiler chicks (Mean ± S.E., n=6).

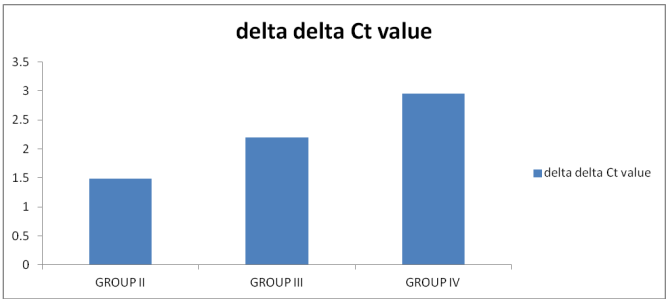

Fig 3:
Effect on HSP 70 expression in terms of delta delta Ct value following daily oral administration of ethion for 30 days in broiler chicks (Mean ± S.E., n=6).

accidental exposure of ethion. It is concluded from the above study that exposure of ethion in broilers for 30 days leads to increased expression of HSP 70 in ethion treated groups. This is an indicative of the stressful conditions exposed following ethion exposure in dose dependent manner.

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Research Article

PERSISTENCE OF HEAVY METALS IN RURAL AREAS OF MATHURA DISTRICT: RISK ASSESSMENT TO ANIMAL HEALTH

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ABSTRACT

Rivers present a potential source of environmental pollution in India. The present study was conducted to assess the degree of heavy metal contamination of surface water, agricultural soil and the fodder crops grown in the catchment area of river, Yamuna in the Mathura district of Uttar Pradesh (India). Surface water, soil and fodder samples were collected from different villages in Mathura District, processed and analyzed for lead, copper, cadmium, mercury, arsenic and iron using Atomic Absorption Spectrophotometer (Perkin Elmer). The results showed presence of all the heavy metals beyond the national and international permissible limits in soil and fodder samples. Levels of arsenic and cadmium in soil (269.83-1891.38 ppb; 11.88–28.26 ppb) and fodder (498.71-6843.84 ppb; 0.82–11.51 ppb) in soil and fodder samples were evident in the villages nearby Mathura oil refinery area suggesting an additional load attributable to the refinery. The health risk index for cadmium (127.88) was remarkably high followed by iron (60.19) and arsenic (31.38) as compared to lead (5.01), copper (0.46) and mercury (0.06). Urgent attention is needed to devise and implement appropriate means of monitoring and regulating industrial and domestic effluent, and develop alternate water resources for surface water for human and animal consumption and agricultural irrigation.

Key words: Heavy metals, Atomic absorption spectrometer, water, fodder, soil

INTRODUCTION

The Yamuna, one of the most sacred rivers in India, flowing through Mathura, presents a picture of total neglect and the river runs like a huge sewage canal transporting industrial effluents and municipal waste. The Yamuna water quality at Mathura stretch is characterized by offensive odour and an ugly look and even at some points, is jet black, with a thick layer of waste scum on the surface, stinking sewage, ûoating dead animals, high load of organic and inorganic material, signiûcant oxygen depletion, excessive presence of pathogens and periodic mass killing of ûsh and other aquatic life. Different heavy metals like lead, cadmium, arsenic, mercury and iron have been implicated with gastrointestinal (GI) disorders, diarrhoea, stomatitis, tremor, hemoglobinuria causing a rust-red colour to stool, ataxia, paralysis, vomiting and convulsion, depression, and pneumonia and have reported to be potent neurotoxic, carcinogenic, mutagenic or teratogenic (McCluggage 1991; European Union, 2002).

Most of the city's wastewater is discharged into the Yamuna upstream of Mathura.Heavy metal accumulation has been identified as the major cause for poor animal health in Mathura area. The sources of heavy metals may be either surface water or soil through fodder. The presence of crude oil refinery in Mathura further contributes through its effluents and waste disposal making the level of heavy metal contaminant an important subject of concern for risk assessment of water, soil and feed/ fodder in this area. Therefore, the present survey was conducted to estimate the concentration of heavy metals viz. copper, lead, cadmium, mercury, iron and arsenic in the surface water, soil and fodder grown in some villages of Mathura district.

MATERIALS AND METHODS

Study sites

The present study was carried out in seven adjoining villages of Mathura district lying in the catchment area of Yamuna namely Salempur, Jachonda, Mukundpur, Bati, Mahavan, Bandi, and Bajna. Out of these selected villages, Salempur and Jachonda were also in close vicinity of Mathura Oil Refinery.

Collection and processing of samples

A total of 147 samples were collected which included 50 samples of soil, 50 samples of water and 47 samples of fodder. The water samples from the available resource viz. ponds, tap water, hand pumps, river etc., feed/fodder samples from available feed/ fodder in season and soil samples from the land adjoining to farmland and water resources were collected. The water samples (n=50) were collected in plastic cans, feed/fodder samples (n=47) in air tight polythene bags, and soil samples (n=50) from the depth of 15-20 cm below the soil surface level. Soil and fodder samples constituted equal amount of the samples from four corners and one central place of the field. Water samples were collected at random from ponds, well and hand–pumps which were the source of drinking water for animals. At least 5–7 samples were collected in plastic vials from each point. Without disturbing the sediments, about 500 ml of water was collected from each

source.

Sample preparation

Water samples (500 ml) were filtered using Whatmans filter paper no. 42 for estimation of dissolved metal content. Filtered water samples (500 ml each) were preserved with 2 ml of nitric acid to prevent the precipitation of metals. Both the samples were concentrated to tenfold on a water bath and subjected to nitric acid digestion using the microwave-assisted technique (pressure 30 psi; 700 watts) (Clesceri, 1998; Anton Paar, 1998).

Soil samples were air-dried and ground into fine powder using pestle and mortar and passed through 1 mm sieve. Soil samples (2 g each) were taken in 250 ml glass beakers and digested with 8 ml of aqua-regia on a sand bath for 2 h. After evaporation to near dryness, the samples were dissolved in 10 ml of 2% nitric acid, filtered and then diluted to 50 ml with Milli-Q water (Chen and Ma, 2001).

Feed/ fodder samples were thoroughly washed first with distilled water and again with millipore water to remove all adhered soil particles. Samples were cut into small pieces, air dried for 2 days and finally dried at 100 ± 1^0C in a hot air oven for 3h. The samples were ground in warm condition and passed through 1mm sieve. Digestion of these samples (2 g each) was carried out using 10 ml nitric acid by Di-acid digestion method, according to the procedure used for soil samples (Lark *et al.,* 2002)

Analysis

Metals analyses were carried out with the help of Atomic Absorption Spectrometer (Model AAnalyst 400, Perkin Elmer, USA), by using flame technique for lead (Pb), iron (Fe) and copper (Cu) and hydride generation (HGA) technique for arsenic (As) and mercury (Hg). For estimation of cadmium, graphite furnace technique was used. In flame technique, air (flow rate 10 l/min) and acetylene (flow rate 2.5 l/min) were used as oxidants/fuel. In HGA technique, the digested sample is diluted to a known volume. An aliquot of the digest is reduced with sodium borohydride to yield metallic ion which was estimated by hot (As) and cold (Hg) vapour atomic absorption spectrometry. Sample analysis was done by using the Win Lab 32 software from Perkin Elmer.

The calibration curves were prepared individually for all the metals by running different concentration of standard solutions with correlation co-efficient (R^2) of 0.998-0.999. At least three known concentrations of the standards (Merck) were used for calibration of the element and then unknown samples were analysed as per calibration (in triplicate) and the concentration of elements was expressed in ppm and ppb levels. The instrument was set to zero by running the respective reagent blanks.

Analysis of data

Daily Dietary Index (DDI)

As fodder crops are contaminated by heavy metals so their daily intake was estimated by the following formula:

DDI =X × Y/ B

Where,

X = metal in vegetable

Y = approximate daily intake of dry wt. of the vegetable

B = average body mass of the consumers

Estimated exposure

Estimated exposure was obtained by dividing daily intake of heavy metals by their safe limits.

Health risk index (HRI)

The health risk index was calculated as the ratio of estimated exposure of test crops and oral reference dose (Cui *et al.* 2004). Oral reference doses were 0.04 and 0.001 mg kg^{-1} day^{-1} for Cu and Cd, respectively, and 0.004 mg kg^{-1} day^{-1} for Pb and 0.009, 0.8 and 0.1 mg kg^{-1} day^{-1} for Fe, As and Hg respectively.

Metal pollution index (MPI)

To examine the overall heavy metal concentrations in all crops analysed in the wastewater irrigated site, metal pollution index (MPI) was computed. This index was obtained as per the method described by Usero *et al.* (1997) by calculating the geometrical mean of concentrations of all the metals in the fodder, water and soil samples.

$MPI(\mu g/g) = Cf_1 \times Cf_2 \times \ldots\ldots \times Cfn)^{1/n}$

where, Cf_n = concentration of metal n in the sample.

RESULTS AND DISCUSSION

The concentrations of heavy metals in water samples collected from different villages ranged between 0.04 – 2.05ppm for Pb, 0.09 – 8.88 ppm for Fe and 0.01 – 0.67ppm for Cu, whereas As, Cd and Hg ranged between 0.71 - 110.34, 0.01 – 2.23 and 0.07 – 5.16 ppb, respectively (Table 1). For all the heavy metals, the highest concentration obtained exceeded the permissible limit set by WHO (1996). Among the heavy metals, the mean concentration was maximum for As (55.20 µg/L) (Fig. 1 & 2).which is more than the safe limit (Awasthi, 2000). Highest heavy metal accumulation was observed in Jachonda and Mukundpur followed by Salempur and Bati which was comparatively nearer to the Yamuna banks as compared to other villages. Out of these four villages showing higher degree of contamination, Salempur and Jachonda were located downstream the Gokul barrage while Bajna was upstream of the other villages. Heavy metals in these villages may be associated with small scale industries such as tannery, electroplating, metal surface treatments, fabric printing, battery and paints, releasing Cd, Cu, Pb, Zn, Ni and other heavy metals into Yamuna through sewage drain. As far as oil refinery is concerned, the treated effluent of refinery is discharged

Table 1.
Range of heavy metals in water

S. No.	Village	Lead (ppm)	Iron (ppm)	Copper (ppm)	Arsenic (ppb)	Cadmium (ppb)	Mercury (ppb)
1	Salempur	0.13-1.29	0.81-7.93	0.04-0.67	7.77-35.25	1.60-2.01	0.31-1.64
2	Jachonda	0.04-2.05	0.83-8.09	0.02-0.49	7.71-32.55	0.02-2.10	0.28-1.32
3	Bandi	1.15-1.21	0.35-5.62	0.01-0.63	0.71-110.34	0.01-2.35	0.24-5.16
4	Mahavan	0.12-1.1	0.09-8.20	0.01-0.28	2.62-8.89	0.15-0.97	0.51-0.63
5	Bajna	0.09-1.14	0.35-8.04	0.01-0.38	3.66-29.19	0.03-2.13	0.07-1.22
6	Mukundpur	0.16-1.01	1.20-8.88	0.02-0.45	0.94-39.09	0.04-1.98	0.26-2.03
7	Bati	0.19-0.93	0.98-8.56	0.03-0.52	3.66-26.19	0.01-2.23	0.37-1.11

Table 2.
Range of heavy metals in soil

S. No.	Village	Lead (ppm)	Iron (ppm)	Copper (ppm)	Arsenic (ppb)	Cadmium (ppb)	Mercury (ppb)
1	Salempur	25.2-68.5	415.6-892.5	3.3-47.9	1776.0-2019	10.5-54.3	26.05-38.2
2	Jachonda	16.3-85.00	402.35-1031	9.7-16.5	288.85-3187	12.2-17.52	7.5-146.15
3	Bandi	15.2-74.6	2.65-1090	20.3-53.9	1832-2055	4.1-23.1	45.85-263.2
4	Mahavan	8.6-52.8	326.7-1074	5.6-12.5	80.7-1959	17.4-19.2	13.55-61.1
5	Bajna	11.5-46.8	425.2-1059.5	4.3-18.4	225.3-1891	8.3-23.6	52.2-68.5
6	Mukundpur	9.85-58.9	846-1091	2.7-13.3	176.65-394	11.23-26.5	23.5-78.9
7	Bati	12.4-69.4	202.23-825.4	1.8-16.4	278.4-1964	6.6-18.7	33.65-66.05

Table 3.
Range of heavy metals in fodder

S. No.	Village	Lead (ppm)	Iron (ppm)	Copper(ppm)	Arsenic (ppb)	Cadmium (ppb)	Mercury (ppb)
1	Salempur	0.7-26.9	322.7-2101	3.3-47.9	639.6-30150	1.0-17.4	59.7-161.8
2	Jachonda	7.5-15.0	371.1-620	17.6-33.6	679.3-15665	2.1-28.8	65.1-229.2
3	Bandi	1.3-7.2	322.3-2103	0.1-10.1	639.6-7440.1	0.1-2.1	21.3-29.3
4	Mahavan	2.2-8.7	139.6-2097	2.6-21.5	848.8-7530	3.0-17.6	77.4-280.7
5	Bajna	0.92-7.6	398.3-2043	0.5-13.5	395.5-700.9	1.3-12.3	24.5-172.6
6	Mukundpur	2.8-11.23	516.5-2111	3.1-28.6	201.3-1001.0	2.7-21.6	35.5-136.2
7	Bati	1.12-9.3	174.3-1963	2.2-32.3	578.0-3929	0.8-8.6	28.3-110.6

Table 4.
Daily dietary index (DDI), Estimated exposure (EE) and Health risk index (HRI) of heavy metals in fodder samples of Mathura district

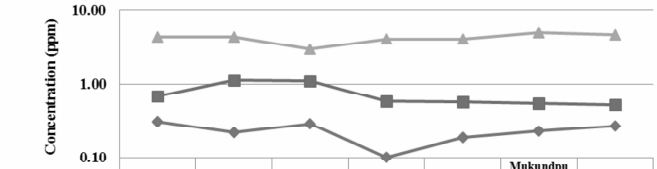
Index	Lead	Copper	Iron	Arsenic	Cadmium	Mercury
DDI	0.16	0.36	27.08	125.51	2.55	0.19
EE	0.02	0.018	0.541	25.102	0.127	0.006
HRI	5.015	0.462	60.191	31.378	127.875	0.065

into Yamuna River at downstream of Mathura city through a lined channel, and into the adjacent Barari Canal, and may be a likely source for Salempur and Jachonda. Otherwise the excessive heavy metal concentration in water samples seems to be associated with the waste material and effluents coming downstream with Yamuna. However, very low levels of these metals, much below the permissible limits; have been reported in the wastewater and surface water at the agricultural farm in the premises of the Mathura Oil Refinery (Hayat *et al.,* 2002).

The concentrations (ppm) of heavy metals in soil samples ranged between 8.6 – 85 for Pb, 2.65 – 1091.0 for Fe and 2.7 – 53.9 for Cu, whereas As, Cd and Hg ranged between 80.7 – 3187.0, 4.1 – 54.3 and 7.5 – 263.2 ppb, respectively (Table 2). The soil samples revealed significantly higher levels as compared to the corresponding water samples. The heavy metal concentrations were again higher than the safe limits of India (Awasthi, 2000) and WHO (1996), suggesting geoaccumulation (Fig 3 & 4). In accordance with this, the soil samples at the farms in vicinity of Oil Refinery revealed a slow and continuous built up of concentrations over years, probably due to fast sedimentation and accumulation in the soil (Hayat *et al.,* 2002).

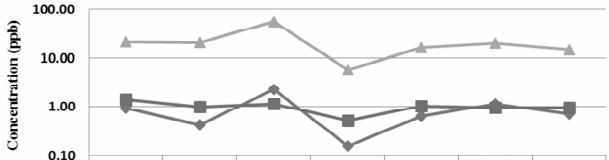
Heavy metal concentrations showed noteworthy variations among samples collected from different villages. The levels were significantly higher in fodder samples (Table 3) as compared to the respective soil and water samples again indicating towards bioaccumulation of these metals in animals through the food chain as observed in case of

Fig 1. Concentration (ppm) of heavy metals in water samples



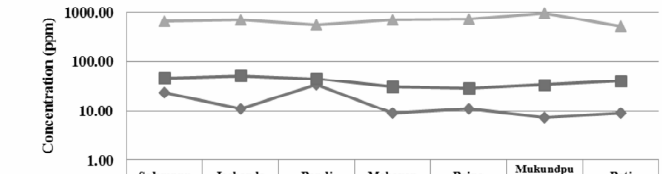
	Salempur	Jachonda	Bandi	Mahavan	Bajna	Mukundpur	Bati
Copper (ppm)	0.31	0.22	0.29	0.10	0.19	0.23	0.27
Lead (ppm)	0.69	1.13	1.11	0.58	0.58	0.55	0.52
Iron (ppm)	4.31	4.40	2.96	4.11	4.10	4.96	4.62

Fig 2. Concentration (ppb) of heavy metals in water samples



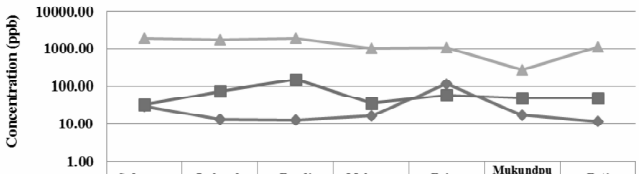
	Salempur	Jachonda	Bandi	Mahavan	Bajna	Mukundpur	Bati
Mercury (ppb)	0.93	0.43	2.24	0.16	0.64	1.13	0.70
Cadmium (ppb)	1.42	0.99	1.15	0.51	1.02	0.96	0.95
Arsenic (ppb)	21.12	20.85	55.20	5.69	16.37	19.94	14.86

Fig 3. Concentration (ppm) of heavy metals in soil samples



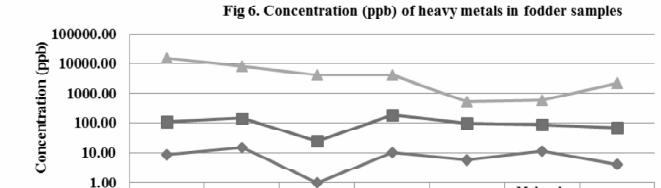
	Salempur	Jachonda	Bandi	Mahavan	Bajna	Mukundpur	Bati
Copper (ppm)	23.58	11.05	34.02	8.95	10.95	7.32	8.91
Lead (ppm)	46.68	50.64	44.84	30.56	29.10	34.31	40.50
Iron (ppm)	648.02	710.58	546.32	698.25	738.31	963.42	509.71

Fig 4. Concentration (ppb) of heavy metals in soil samples



	Salempur	Jachonda	Bandi	Mahavan	Bajna	Mukundpur	Bati
Cadmium (ppb)	30.35	12.86	12.60	16.30	113.95	16.86	11.65
Mercury (ppb)	32.08	74.72	148.39	35.12	58.25	49.15	47.88
Arsenic (ppb)	1891.53	1725.92	1934.50	1014.85	1052.15	278.33	1117.20

Fig 6. Concentration (ppb) of heavy metals in fodder samples



	Salempur	Jachonda	Bandi	Mahavan	Bajna	Mukundpur	Bati
Cadmium (ppb)	8.74	14.85	1.01	9.93	5.75	11.05	4.07
Mercury (ppb)	110.75	147.15	25.30	179.05	98.55	85.85	69.45
Arsenic (ppb)	15374.30	8168.08	4029.31	4178.21	545.15	600.05	2248.60

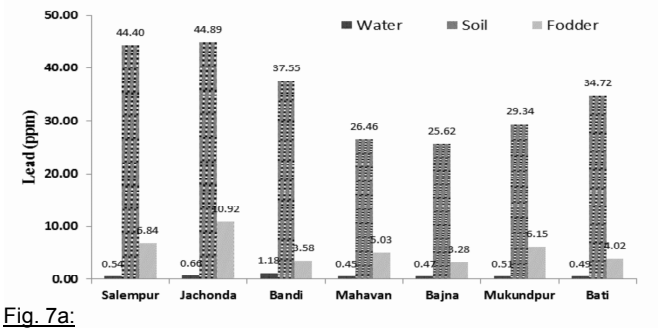


Fig. 7a:
Metal pollution index (MPI) of Lead

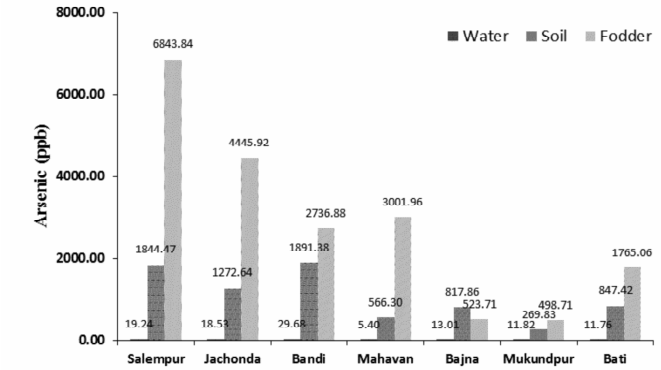


Fig. 7b:
Metal pollution index (MPI) of arsenic

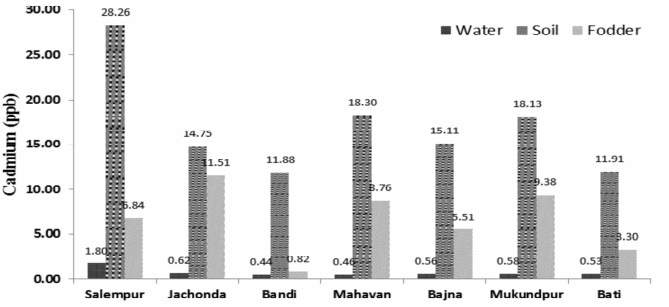


Fig. 7c:
Metal pollution index (MPI) of cadmium

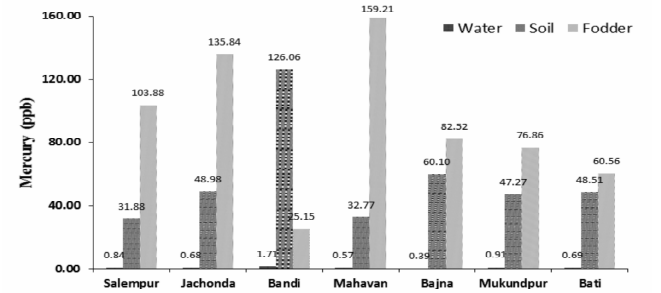


Fig. 7d
Metal pollution index (MPI) of mercury

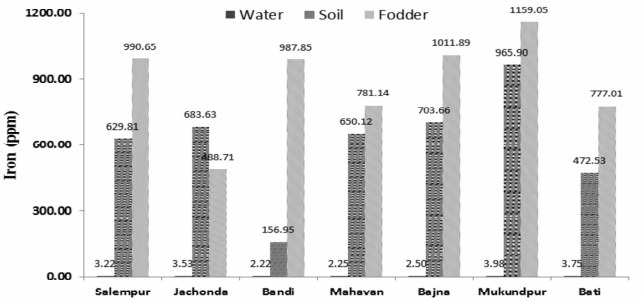


Fig. 7e:
Metal pollution index (MPI) of iron

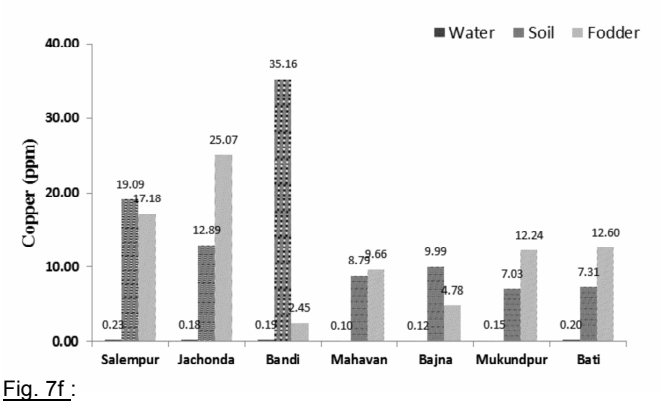


Fig. 7f :
Metal pollution index (MPI) of copper

man (Muchuweti *et al*., 2006). A significant accumulation of As, Hg and Fe was observed in feed/fodder samples whereas, Pb and Cd did not show accumulation in plants while copper showed no specific relationship of accumulation from water to soil to plants.

Once the mercury comes in the environment, it is transformed by soil bacteria into methyl-mercury and then it bioaccumulates in flora and fauna as observed in the present study. The highest level (15374.30 ppb) of arsenic was recorded from Salempur village followed by Jachonda, Mahavan and Bandi (Fig 5 & 6). Exposure to arsenic is thought to be caused mainly through arsenic-contaminated underground water and use of such water for field irrigation enhances the possibility of arsenic uptake into crop plants. In general, arsenic content in plants varied considerably with type of plants, type of soil, and arsenic content of irrigation-water. Earlier workers have also reported accumulation of higher concentrations at panicle initiation stage in the plants grown on heavily arsenic contaminated soil (Rahman *et al.,* 2008). The comparison of arsenic content in soil with arsenic content of feed/fodder revealed significant relationship, indicating that levels of arsenic in soil dictated the arsenic uptake capacity of plants. Very high concentrations observed in Salempur are indicative of a special capacity of the plants to bio-accumulate arsenic. The highest concentrations of arsenic were always recorded in roots of the fodder plant, and this may be attributed to contamination from fine colloidal particles. Earlier studies have also revealed that some grasses, used as fodder, accumulated arsenic at higher concentration than that of soil (Imamul Haq *et al.,* 2006) as observed in the present study. Significant levels of iron were observed in water, soil and feed/fodder in the present study. Iron has previously been reported to accumulate in leaves with chloroplast holding upto 80% of metal content in the plants grown on contaminated soil (Briat *et al.,* 2006).

The permissible limit of lead in plants, as recommended by WHO, is 5-8 ppm. Accumulation of lead in soil is a widespread issue. It enters the food chain and accumulates with age in different tissues like bones, aorta, kidney, liver and spleen and interferes with the normal physiological processes. It comes in the environment mainly in the form of dust. Although it was found to be present in water and soil samples at appreciable levels, comparatively lower concentration of lead in plants were perhaps due to reason that, prior to processing of samples, the green fodder samples were washed and the lead present in the form of dust might have been washed off. Among all heavy metals, least accumulation of cadmium was observed in plants. The maximum level that has been observed in most of the plant species grown in non-industrial area is 3 ppm and 12 ppm in the plants grown in the vicinity of industrial smelters (Leach *et al.,* 1979). The permissible limit of cadmium in plants, as recommended by WHO, is 0.02 mg/kg. In almost all the plant samples it was more than the permissible limits. Even the borderline values indicate a sign of danger for the animals and people living in those areas because it is a toxic heavy metal with a very long tissue life but no beneficial effect. Cadmium levels in crops depend on uptake from soils and the rate of uptake is influenced principally by the form of the element, the soil, physico-chemical properties and the plant species (Scientific opinion, 2009). Cadmium enters the body via the gastrointestinal tract by eating food products grown on contaminated soil. Some phosphate fertilizers contain cadmium and may contribute to an increase in the concentration of cadmium in soil. Cadmium intoxication becomes detectable when it exceeds the threshold level of 5070 mg/ day.

The permissible limit of copper for plants is 10 - 20 ppm recommended by WHO and all the plant samples from different villages have copper contents within this range except Salempur (24.96 ppm) and Jachonda (24.62 ppm). Most copper compounds have a tendency to settle and remain bound to water, sediments or soil particles. Soluble copper compounds pose a threat to animal and human health. Usually water soluble copper compounds occur in the environment through application in agriculture (Iqbal *et al.* 2011).

The metal pollution index for different heavy metals has been illustrated in Fig. 7a-7f. In present study, it has been observed that water, soil and feed/fodder samples were heavily polluted with lead (Pb), arsenic (As), cadmium (Cd), mercury (Hg), iron (Fe) and copper (Cu) in almost all the villages compared to WHO maximum permissible limits (WHO, 1996). For all the six metals considered for the present study, the concentration was found highest in feed/ fodder followed by soil and water. A significant accumulation of As, Hg and Fe was observed in feed/fodder samples whereas Pb and Cd did not show accumulation in plants while copper showed no specific relationship of accumulation from water to soil to plants.

The accumulation of heavy metal in plants is well documented. These heavy metals come in the environment

owing to different manmade processes in the form of industrial waste/effluents and enter the water bodies (ecosystem) where it may persist as such or get modified into an another chemical form. Transformation of the inorganic form of mercury to organic form leading to bioaccumulation in flora and fauna is well proven. These heavy metals accumulate over time in soil and their subsequent migration to crops is influenced by pH, redox potential and type and quality of the soil. One of the most important environmental issues is the level of groundwater contamination with metals/metalloids because of their toxicity at even minor levels (Wongsasuluk *et al.,* 2013).

Daily Dietary Index, estimated exposure and health risk index are suggested to be a reliable and precise method for monitoring of metal pollution/accumulation in living organism owing to water resources (Usero *et al.*, 1997) and results of present study are illustrated in Table 4. Consumption of foodstuff with elevated levels of heavy metals may lead to high level of accumulation in the body causing related health disorders. To assess the livestock health risk associated with heavy metal contamination of plants grown in these areas, estimated exposure and risk index were calculated. The accumulation of heavy metals is ordered by their relative affinity to form oxides and hydroxides at neutral or slightly basic pH (Stengel and Gelin, 2004). The irrigation by waste water generally leads to accumulation of the heavy metals in agricultural soils but the higher soil pH thwarts their uptake to the fodder plants (Iqbal *et al.* 2011).Variations in the heavy metal concentrations between the fodder samples collected from different villages reflect the differences in uptake capabilities and their further translocation to the edible portion of the plants. The concentrations of all the heavy metals were significantly above the national and various international permissible limits in all the fodder crops. The high dietary index and health risk index of heavy metals also suggest that arsenic, cadmium and iron contamination in most of the test plants has potential for animal health risk. The health risk index for cadmium was remarkably high followed by arsenic and iron as compared to lead, copper and mercury. To add to this, the cows fed on cadmium rich fodder have been found to show high cadmium levels in their milk (Lokeshwari and Chandrappa, 2006). Consumption of this milk for a long period may pause reasonable human health risk. The reason for Cd accumulation in fodder crops might be due to relative ease of uptake by leafy plants. The foliage may also show an additional absorption of atmospheric deposits on leaves (Mido and Satake, 2003). The absorption from atmospheric deposits may also be a factor for lead accumulation in the fodder.

The study suggests that even though there are low concentrations of certain heavy metals in surface water, its long term exposure to soil may cause heavy metal contamination leading to health risk of humans and animals. Thus urgent attention is needed to devise and implement appropriate means of monitoring and regulating industrial and domestic effluent, and develop alternate water resources for surface water for human and animal consumption and agricultural irrigation.

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Short Communication

ANTIPYRETIC EFFECT OF HOT METHANOLIC LEAVES EXTRACT OF *CALOTROPIS GIGANTEA* ON BREWER'S YEAST INDUCED PYREXIA

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ABSTRACT

Investigations were conducted to validate the folklore medicinal claims of use of Calotropis *gigantea* (madar) in fever. The antipyretic activity of the extract was evaluated in lowering the Brewer's yeast-induced pyrexia in comparison to the reference antipyretic aspirin in rats. The per cent reduction in pyrexia among aspirin-treated rats varied between 76.21 and 86.81 per cent, and among the extract-treated rats (100 to 1000 mg/kg) from 14 to 60 per cent up to 6 hr of post-treatment. The methanol extract of its leaf powder (100, 300 and 1000 mg/kg PO) was found to exhibit significant and dose – dependent antipyretic effect in brewer's yeast induced – pyrexia in Wister rats. However, the extract at 1000 mg/kg oral doses had no effect on the normal body temperature of rats.

Key words: *Calotropis gigantea*, leaves extract, fever, brewer's yeast.

The plant *Calotropis gigantea* (milk weed) belonging to the family Asclepiadaceae locally popular as madar folklore medicine is one of the important indigenous medicinal plants of India. All parts of the plants including the latex have been recognized to possess varied therapeutic potentials, including as a remedy in fever (Nadkarni, 2001; Oudhia, 2001) and anthelmintic activity (Saravanpriya and Sivskumar, 2005) and antibacterial activity (Muniruzzaman and Choudhary, 2004). Recently the latex of the plant has been reported clinically beneficial in clearing corneal opacity in two cross- bred cows (Hegde and Nimbalkar, 2003) and the hydroalcoholic extract of its aerial parts to possess antidiarrhoeal and analgesic properties (Chitme *et al*., 2004a: 2004b). In view of validating the traditional claims of its use in treating fever the present investigation was undertaken to evaluate the antipyretic activity of *C. gigantea* leaves.

Fresh mature leaves, in bulk, were locally obtained from a single *C. gigantea* plant. The leaves were cleaned and shade-dried under a fan at room temperature. The dried leaves were ground into a fine powder with the help of an electrical grinder. The powder was processed to obtain methanol extract using Soxhlet's extraction.

Antipyretic activity of the methanol extract was determined in male Wistar rats against Brewer's yeast induced pyrexia (Loux *et al*., 1972). Thirty six rats weighing between 130 and 180 gm were randomly assigned to six groups, each comprising of six animals. The Group I rats served as extract control, which were orally administered the extract at the maximum dose level of 1000 mg/kg. The II Group animals served as pyrexia control group, where the rats were administered the pyrogen Brewer's yeast and at 18 hr thereafter the normal saline. The Group III rats were treated with Brewer's yeast and at 18 hr thereafter the reference antipyretic aspirin @ 300mg/kg orally. The Groups IV, V and VI rat were administered with Brewer's yeast and at 18 hr thereafter the extract at 100, 300 and 1000 mg/kg respectively. Body temperature of each rat was recorded with the help of a property lubricated clinical thermometer by inserting approximately 4 cm into the rectum for 45 sec. immediately before Brewer's yeast injection, at 18 hr after post Brewer's yeast injection (0 hr) and subsequently at 1, 3 and 6 hr of post drug/ extract administration. The pyrexia was determined from the difference between 0 hr and -18[a] body temperature. Body temperature taken at 0 hr served as the pre- drug pyrexia for each group. The percent inhibition in pyrexia was determined following each treatment at different post-treatment intervals. The results of study were interpreted by using paired t - test (Snedecor and Cochran, 1967).

The observations and results of this experiment are reproduced in Table 2. The mean normal rectal temperature among the rats in all the six groups was statistically similar, which was in the range of 99.35 ± 0.06 to 99.60 ± 0.04 ºF. The rectal temperature in the groups (II to VI) which received the Brewer's yeast was elevated (pyrexia temperature) which ranged between 100.68 ± 0.12 and 101.20 ± 0.06 ºF. The difference in rectal temperature between the respective two intervals in all the groups was significant ($P<0.01$). Subsequent to aspirin administration in Group III the temperature was significantly lowered ($P<0.01$) to 99.98 ± 0.04, 99.86 ± 0.04 and ºF at 1, 3, and 6 hr of post – treatment, respectively. The temperature in Group IV rats, which

TABLE 1:
Antipyretic effect of hot methanolic extract of *Calotropis gigantea* leaves in rats.

Group No.	Treatment	Mean Rectal Temperature (°F) ± SE				
		Normal	Pyrexia *	Post-Asprin Treatment		
				1 hr	3 hr	6 hr
I	Extract @ 1000 mg/kg	99.45 ± 0.06	99.53± 0.04	99.45 ± 0.03	99.32 ±0.09	99.46 ±0.06
II	Brewer's yeast (BY)	99.60 ± 0.04	101.20 ± 0.06[a]	101.20 ±0.14	101.36 ±0.14	101.18 ± 0.10
III	BY + Aspirin @ 300 mg/kg	99.49 ±0.05	101.20 ± 0.06[a]	99.98 ± 0.04[b](76.21)	99.86 ± 0.04[b](83.00)	99.81 ± 0.03[b](86.81)
IV	Extract @ 100 mg/kg + BY	99.52 ± 0.06	100.80 ± 0.11[a]	100.61 ±0.06(14.00)	100.59 ±0.06(16.03)	100.57 ± 0.07(17.55)
V	Extract @ 300 mg/kg + BY	99.52±0.06	100.68 ± 0.12[a]	100.45 ± 0.10[b](19.82)	100.30 ± 0.11[b](32.00)	100.15 ± 0.11[b](45.68)
VI	Extract @ 1000 mg/kg + BY	99.35 ± 0.06	100.85 ± 0.05[a]	100.00 ± 0.05 [b](56.67)	99.95 ± 0.05[b](60.00)	99.93 ± 0.05[b](61.33)

a : Significantly elevated as compared to normal temperature (P < 0.01), b : Significantly lowered as compared to pyrexic temperature (P < 0.01), * : After 18 hr of injection of Brewer's yeast, Figures in parentheses indicate per cent decrease from pyrexic temperarure BY: Brewer's yeast

received the extract at 100 mg/kg at the three post-treatment intervals ranged between100.57 ± 0.07 and 100.61 ± 0.06 ºF, which was statistically similar to the pyrexia temperature of 100.80 ± 0.11 ºF . The pyrexic rats in Groups V and VI which received the extract at 300 and 1000 mg/kg respectively, showed the mean temperatures of 100.15 ± 0.11 to 100.45 ± 0.10 ºF and 99.93 ± 0.05 to 100 ± 0.05 ºF respectively during the post- treatment. The reduction in temperature from that at pyrexic level in both these groups was significant (P< 0.01). Accordingly, the per cent reduction in pyrexia in aspirin treated rats varied between 76.21 and 86.81 in aspirin - treated rats: whereas, the reduction in rectal temperature among the extract – treated three groups (IV, V and VI) varied from 14 to 17.55, 19.82 to 45.68 and 56.67 to 61.33 per cent, respectively. The antipyretic effect tended to persist up to or beyond 6 hr of post- treatment at 1000mg/kg dose of the extract similar to aspirin. The pyrexic rats (Group II), which neither received the antipyretic aspirin nor the extract continued to show elevated rectal temperature during the observation period (101.18 ± 0.01 to 101.36 ± 0.14 ºF). Similarly, the Group I rats which received the maximum dose of the extract (1000mg/kg) continued to show normal temperature, indicating that the methanol extract of C*. gigantea* leaves had to effect on the normal body temperature.

The antipyretic activity of methanol extract of *C. gigantea* leaves powder is supported by a very recent report of Chitme *et al.,* (2005), who observed antipyretic effect of *C. gigantea* root extracts (at 200 and 400mg/kg intra- peritoneal doses) against yeast – or typhoid vaccine induced pyrexia in rats and rabbits. The traditional use of *Calotropis* plants in fever has also reported by various works.

The results of the present investigation it is evident that the methanol extract of C*. gigantea* leaves did possess dose dependent antipyretic effect.

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