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Review Article

RESISTANCE AGAINST FLUOROQUINOLONES IN *E. COLI*: ROLE OF EFFLUX PUMPS

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ABSTRACT

Emergence of bacterial strains resistant to antimicrobials is a matter of great concern worldwide. Fluoroquinolones have been extensively used in human and veterinary medicine due to their effectiveness against both gram-positive and gram-negative bacteria, however, in recent years, resistance to fluoroquinoloneshas increased globally, particularly in the members of *Enterobacteriaceae*. Resistance against fluoroquinolones is multifactorial, with rates of resistance vary by both organism and geographic region. Main mechanisms of resistance include one or a combination of target-site gene mutations (Topoisomerase II and Topoisomerase IV), increased production of multidrug-resistance (MDR) efflux pumps, modifying enzymes, and/or target-protection proteins. Fluoroquinolone-resistant clinical isolates of bacteria have emerged readily worldwide and an understanding of the multiple mechanisms of resistance may help in fighting the spread of resistance against these drugs. Here, we outline various mechanisms of fluoroquinolone resistance against *E. coli* with special reference to the over expression of AcrAB-TolC efflux pumps. Determining the role of efflux pumps in resistance will greatly contribute to limiting the resistance development.

Key word : *E.coli*, efflux pump, fluorpquinilones, gene expression, multidrug resistance.

INTRODUCTION

Antimicrobial resistance is one of the most pressing global concerns in both human and veterinary medicine. The World Health Organization has urged to "evaluate the strategies to overcome and control the spread of antimicrobial resistant micro-organisms" (World Health Organization, 2017). Although there are concerns about the emergence of quinolone-resistant bacterial strains in humans and animals but still fluoroquinolones are amongst the most commonly used drugs for the treatment of mild to severe bacterial infections. Thus it is imperative to judiciously use fluoroquinolones to preserve their effectiveness. In veterinary medicine, it is essential to reserve these drugs for cases requiring a powerful antibacterial effect and to administer them exclusively after thorough clinical assessment with appropriate regimens (Trouchon and Lefebvre, 2016), because the selection of improper dose and dose intervals are accelerating the resistance against these drugs. As current therapeutic options are scarce to deal with resistant organisms, therefore, a thorough understanding of the mechanisms through which resistance develops has become critical to fight the spread of antimicrobial resistance.

FLUOROQUINOLONES

The quest for new antimicrobial drugs with greater potency, improved safety and broader spectrum of activity has led to the introduction of many new drugs. An important addition to the drug armamentarium is the fluoroquinolone group. Fluoroquinolones, as their name suggest, are derived from the quinolone family of antimicrobials. Quinolones themselves are synthetic constructs, developed by modification of 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid (Redgrave *et al* 2014). The first member of quinolone anti-bacterials to be introduced was Nalidixic acid in mid-sixties and its congener oxalones were introduced in 1970s. Increased spectrum was achieved by introduction of fluoroquinolones in 1980s. Fluoroquinolones are produced by fluorination of the basic quinolone structure at 6th position whereas introduction of a piperazine substitution at 7th position results in production of different derivatives having higher potency, expanded spectrum and improved pharmacokinetics and slow development of resistance (Ball, 2000). Enrofloxacin was the first fluoroquinolone introduced into veterinary medicine (Papich, 1999). These compounds have now been used in clinical practice for over two decades, and during this time an increased understanding of structure-activity relationships of the fluoroquinolones has led to the development of even better compounds in terms of both the spectrum of antimicrobial cover and improved pharmacokinetics, allowing once-daily dosing and use as a monotherapy (Emmerson and Jones, 2003). Marbofloxacin is a carboxylic acid derivative, third generation fluoroquinolone, developed exclusively for the use in veterinary medicine (USP, 2007).

MECHANISM OF ACTION OF FLUOROQUINOLONES

Fluoroquinolones target DNA gyrase and topoisomerase IV with varying efficiency in different

bacteria and inhibit their control of supercoiling within the cell, resulting in impaired DNA replication (at lower concentrations) and cell death (at lethal concentrations) (Drlica *et al.,* 2009). The targeting of either DNA gyrase or topoisomerase IV as the primary target by fluoroquinolones varies with bacterial species and specific fluoroquinolone; however, as a broad generalization, the key target in gram-negative bacteria is DNA gyrase, whereas in gram-positive microorganisms topoisomerase IV is preferentially targeted. When either DNA gyrase or topoisomerase IV induces transient double-strand DNA breaks, they first bind covalently to the DNA to form enzyme–DNA complexes before breaking the bound DNA, passing another segment of DNA through this break, and rejoining the original DNA segment. When a fluoroquinolone is present, the complex is altered into a drug–enzyme–DNA complex (known as a ternary complex) in which type II topoisomerase is trapped with the bound DNA (Drlica, *et al.,* 2008). The basis of the interaction of quinolone with topoisomerase IV is the formation of a water–metal ion bridge between the oxygen molecules in the amine group of the drug and the hydroxyl residues in conserved serine or acidic residues in the enzyme, mediated by a Mg^{2+} ion (Sissi *et al.,* 2013). The binding of fluoroquinolones occurs within the enzyme at the target site of helix-4 in either GyrA or ParC. Fluoroquinolones bind to DNA gyrase or topoisomerase IV, which is then unable to re-ligate the DNA substrate; the broken segments of DNA bound to the enzyme are referred to as cleaved complexes. The formation of cleaved complexes is a reversible process *in vitro* and a pivotal point in the fluoroquinolone killing pathway, which can follow one of two irreversible courses depending on the molecule. The two main routes of lethal fluoroquinolone action are the protein synthesis-dependent pathway (also known as the chloramphenicol-sensitive pathway, due to chloramphenicol's ability to inhibit fluoroquinolone-mediated cell death) and the protein synthesis-independent pathway (the chloramphenicol-insensitive pathway) (Drlica *et al.,* 2008, Drlica *et al.,* 2009). Older first-generation quinolones such as nalidixic acid work via the former mechanism, whereas newer fluoroquinolones largely work via the later pathway, although both result in fragmentation of chromosomal DNA and ultimately cell death.

MECHANISMS OF FLUOROQUINOLONE RESISTANCE IN *E. COLI*

The selection of resistance by an antibacterial agent is an important pharmacodynamic characteristic to evaluate, as this can impact the effectiveness of the drug in clinical practice (Rybak, 2006). In veterinary as well as human medicine, the first aim of antibiotic therapy is to eradicate the pathogens from diseased bodies. However, antimicrobial therapies also need to minimise the risk of resistance in pathogenic or commensal bacteria in order to keep these treatments of veterinary pathologies as efficacious as possible over time and to avoid the spread to human beings through zoonotic bacteria. This is particularly true for the veterinary use of critical antibacterials such as fluoroquinolones. The potent antimicrobial activity and broad spectrum of fluoroquinolones may be beneficial in eliminating pathogens, however, they may adversely suppress microorganisms in normal enteric flora to various degrees which may lead to emergence of antimicrobial resistance in the endogenous gut flora (Donskey, 2004). *Escherichia coli* is one of the most frequently encountered bacterial species of animal and human commensal intestinal flora (Mcdonald *et al.,* 2001) and it has been established that resistance to fluoroquinolones develops more rapidly in *E. coli* than in other members of *Enterobacteriaceae* (Gales *et al.,* 2000). Pathogenic *E. coli* strains are a common cause of infection in humans and animals responsible for intestinal infections and septicemia (Webber and Piddock, 2001). Resistance against fluoroquinolones is complex and several mechanisms have been known to account for the emergence of fluoroquinolone resistance in *E. coli*.

Quinolone resistance determining region (QRDR)/ target-site mutations

In *E. coli*, mutational alteration in the fluoroquinolone target enzyme, namely DNA topoisomerase II, also named DNA gyrase (*gyrA* and *gyrB*), and Topoisomerase IV (*parC*and*parE*), are recognized to be major mechanisms through which resistance develops. The region where mutational hot spots are localized, which encodes fluoroquinolone resistance, is a short DNA sequence known as the quinolone resistance-determining region (QRDR). This region occurs on the DNA binding surface of the enzyme. Mutations in the QRDR of these genes, resulting in amino acid substitutions, alter the target protein structure and subsequently the fluoroquinolone-binding affinity of the enzyme, leading to drug resistance (Redgrave *et al.,* 2014). Although fluoroquinolones preferentially target either DNA gyrase or topoisomerase IV, they will bind to the secondary target and exert an antibacterial effect even if the primary target has been mutated to a resistant allele (Moon *et al.,* 2010). Alteration of the primary target site can be followed by secondary mutations in lower-affinity binding sites and highly resistant organisms will typically carry a combination of mutations within *gyrA* and *parC* in gram-negative organisms. In isolates displaying fluoroquinolone resistance, primary mutations in the QRDR commonly presents substitutions at amino acid position Ser83 and/or Asp87 of the GyrA subunit, while

substitutions at residues Ser80 and Glu84 are commonly identified alterations in the ParC subunit of the topoisomerase IV (Karczmarczyk *et al.,* 2011, Kim *et al.,* 2012); these mutations alter the target site structure and reduce the binding efficiency of fluoroquinolones.

Transmissible quinolone-resistance mechanisms

Although several elegant reviews focus on the chromosomally-mediated resistance mechanisms, however the discovery of the plasmid-borne quinolone resistance determinants has broadened our concerns regarding transmissible mechanisms of quinolone resistance. Various genes encoding different resistance mechanisms and on mobile genetic elements can decrease susceptibility to fluoroquinolone antibiotics; these are often encoded on plasmids and known as plasmid-mediated quinolone resistance (PMQR) genes. Although the acquisition of PMQRs results in only low level fluoroquinolone resistance, they are increasingly being identified worldwide, especially among *Enterobacteriaceae* (Pons *et al.,* 2013, Roderova *et al.,* 2017). The prevalence of PMQR genes among non-pathogenic microorganisms is of special concern as horizontal transfer of resistance would facilitate the rapid dissemination of the resistance genes even to animal and/or human pathogens.

The first PMQR gene to be identified was named *qnr,* found on a plasmid in a clinical isolate of *K. pneumoniae* (Martý´nez *et al.,* 1998). The protein it encodes is characterized by a pentapeptide-repeat motif and has similarities to immunity proteins such as McbG, a protein that confers immunity to the DNA replication inhibitor microcin B17 (Garrido *et al.,* 1988). Data from a recent structural analysis of a Qnr protein suggested that resistance to fluoroquinolones is achieved by the binding of the Qnr protein to the topoisomerase, which physically prevents the intercalation of the antibiotic with the target enzyme (Xiong *et al.,* 2011), however the exact mechanism of DNA gyrase protection conferred by Qnr has yet to be established. Subsequently, *qnr* was later renamed *qnrA*and families of *qnr* genes *(qnrB, qnrS, qnrC,* and *qnrD*) have been described (Robicsek *et al.,* 2006a). The *qnr* genes generally confer modest protection against fluoroquinolones; for example, when the original *qnr*plasmid was transferred into *E. coli* J53, a 16-fold increase in the minimum inhibitory concentration (MIC) of ciprofloxacin was observed (Martý´nez *et al.,* 1998). Robicsek *et al.* (2006a) reported that *invitro* acquisition of a Qnr determinant through conjugation confers a 16 to 125-fold increase in the MIC, depending on the nature of the donor strain and the quinolone tested. However, there is a wide range of MIC changes reported as a result of carriage of *qnr*genes, this has been attributed to differences in plasmid copy number and gene expression (Briales *et al.,* 2012).

After discovery of Qnr proteins, another mechanism of transferable quinolone resistance was reported i.e. enzymatic inactivation of certain quinolones. The *cr* variant of *aac(6')-Ib* gene encodes an aminoglycoside acetyl transferase that confers reduced susceptibility to ciprofloxacin by acetylating the amino nitrogen on the piperazinyl substituent present in these drugs (Martýnez *et al.,* 1998, Robicsek *et al.,* 2006b). This gene harbours two individual base pair substitutions leading to two amino acid changes, Trp102Arg and Asp179tyr, which result in the enzyme being able to acetylate ciprofloxacin and norfloxacin, reducing the activity of the fluoroquinolone and therefore increasing MIC two to fourfold (Robicsek, 2006b).

The third class of mobile fluoroquinolone-resistance genes includes *qepA*and *oqxAB* efflux systems, which encode transporters that can export fluoroquinolone molecules. Carriage of these genes again confers modest increases in the MIC of fluoroquinolones (Strahilevitz *et al* 2009). These genes encode transporters that can export fluoroquinolone molecules**.** The *qepA*was first identified in an *E. coli* isolated from Japan (Yamane *et al.,* 2007) and later found also in an *E. coli* isolated in Belgium (Perichon *et al.,* 2007). *qepA* encodes an efflux pump belonging to the major facilitator subfamily (MSF) (Yamane *et al.,* 2008). The MICs of norfloxacin, enrofloxacin and ciprofloxacin were 32 to 64 fold higher for the experimental strains expressing QepA compared with host strain (Yamane *et al.,* 2007). Recently a plasmid-encoded efflux pump, OqxAB, was discovered in *E. coli* isolates of porcine origin (Hansen *et al.,* 2007). OqxAB was encoded by the genes *oqxA* and *oqxB* located on a 52-kb conjugative plasmid designated pOLA52 and conferred resistance to multiple agents, including fluoroquinolones (Hansen *et al.,* 2007).

Efflux pumps mediated quinolone resistance

In gram-negative bacteria, besides the acquired resistance by the acquisition of external resistance determinants or mutations in genes that code for the drug targets, the intrinsic drug resistance also plays an important role in the resistance towards antibiotics and biocides (Piddock, 2007, Viveiros *et al.,* 2007, Nikaido and Pagès, 2012). This resistance occurs as a consequence of the (i) presence of an outer membrane that create a permeability barrier reducing the influx of antimicrobials, and (ii) overexpression of efflux pumps that help to reduce the intracellular level of antimicrobials and toxins (Nikaido and Pagès, 2012). Effux pumps reduce the concentration of their substrates from within a cell by actively exporting them from the cytoplasm to the external media. These include transporters of various classes; for example, the major facilitator superfamily (MFS) pump NorA of *S. aureus* and the resistance nodulation division (RND) family of tripartite transporters

of gram-negative pathogens (Machado *et al.,* 2017). The efflux pumps of the RND (resistant nodulation cell division) superfamily have been associated with multidrug resistant phenotypes in gram-negative pathogens. The substrates of the RND efflux pumps are different in their structure and physicochemical properties and include antibiotics, detergents, and biocides (Li and Nikaido, 2009). The clinical implication of this substrate promiscuity is the development of multidrug resistance. The major RND efflux system of *E. coli* consists in a typical tripartite efflux pump, the AcrAB-TolC. This structure is composed of: (i) AcrB, an inner membrane protein that functions as the transporter component of the pump; (ii) TolC, the protein that traverses the cell envelope and provides a conduit to the exterior; and (iii) AcrA, two periplasmic embedded membrane proteins that anchor TolC to the plasma membrane (Du *et al.,* 2014). Upon entering in the cell, the compounds will interact with the substrate-binding pocket of AcrB, which will extrude the compounds via TolC using the energy produced by the proton motive force (PMF). The AcrAB activity and overexpression have been associated with the resistance to fluoroquinolones, chloramphenicol, tetracycline, β-lactams, and β-lactamase inhibitors, among others, as well as biofilm formation and pathogenicity (Piddock, 2007). De-repression of *acrAB* results in slightly decreased susceptibility (2 to 4-fold increase in MIC) to fluoroquinolone on its own, but this resistance mechanism combined with QRDR mutations lead to high-level quinolone resistance (Mazzariol *et al.,* 2000). Beside the AcrAB-TolC efflux pump, other *E. coli* efflux systems also play a role in the development of drug resistance (Viveiros *et al.,* 2007), however, *acrAB*over-expressing strains appear to be common amongst highly resistant human and veterinary isolates of *E. coli* (Toyotaka *et al.,* 2013, Sarah and Rania, 2017). Studies of ciprofloxacin resistant isolates from humans and animals found 90 % and 31 % over-expressed *acrA* or *acrB,* respectively (Mazzariol *et al.,* 2000). The incidence of efflux in clinical and veterinary isolates of *E. coli* suggested that it may be an important factor in quinolone resistance (Toyotaka *et al.,* 2013, Sarah and Rania, 2017).

EFFLUX PUMP INHIBITORS

It is reported that combating the resistance could be done by targeting the mechanisms responsible for it. As efflux pumps provide both innate and higher-level resistance to antimicrobials in bacteria, efflux pump inhibitors (EPIs) should ideally increase the activity of antimicrobials in resistant organisms (Lomovskaya *et al.,* 2001). The EPIs can effectively increase the intracellular concentration of the drug to the level essential for its activity and hence reduce the minimal inhibitory concentration required for the antibiotic to kill the resistant organisms. The two main EPIs phenylarginine-β-naphthylamide (PAβN) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) are usually used for the demonstration of an RND type efflux pump of gram-negative bacteria. The peptidomimetic PAβN, the first EPI identified, is active against several RND pumps in a variety of gram-negative pathogens. This compound was originally identified in a screen for potentiators of the fluoroquinolone levofloxacin in the RND pump (MexAB-OprM) expressing organism *Pseudomonas aeruginosa* (Lomovskaya *et al.,* 2001) and is now routinely used to evaluate the contribution of efflux in various gram-negative bacteria (Pages *et al.,* 2005). It is one of the most known efflux inhibitors of the AcrAB efflux pump of *E. coli* and has a competitive mechanism of inhibition (Martins *et al.,* 2009). Recently, Misra *et al.* (2015) showed that PAβN acts as an inhibitor of the AcrAB and AcrEF efflux systems at low concentrations, and as a membrane-destabilizing agent when used at higher concentrations. CCCP is a protonophore and it inhibits the processes dependent upon the energy provided by proton motive force, including active efflux either directly (e.g. AcrAB–TolC) or indirectly via the generation of ATP (Coldham*et al* 2010). It is an energy-dependent EPI that de-energizes membranes unlike PAβN which is more substrate specific. CCCP is not exactly termed as an EPI because it is involved with the proton motive force that is necessary for the working of RND type pumps thereby indirectly inhibiting the efflux mechanism (Hirakata *et al.,* 2009). However, both these compounds are not applicable to clinical use due to their toxic properties.

Several methods have been used to measure the activity of bacterial efflux pump systems. Most of the methods use a molecule that is a substrate of the efflux pump under investigation and whose relative concentration can be easily detected, for example, by measuring fluorescence. The methods can be split into two categories, (i) those that directly measure efflux i.e. how much the substrate is pumped out, and ii) those that measure how much of a substrate molecule accumulates inside the cell, the level of which is then used to infer efflux activity indirectly (Blair and Piddock, 2016). Both types of method typically use dyes, mainly ethidium bromide, that have differential fluorescence when intra or extracellular. Ethidium bromide is a DNA-intercalating agent and its fluorescence strongly increases when bound to DNA. Therefore, fluorescence is higher when intracellular than when extracellular, and this is used to measure the amount of accumulation (Blair *et al.,* 2015). Moreover, the accumulation of ethidium bromide inside the bacterial cells can be increased in the presence of efflux pump inhibitors

(Pages *et al.,* 2005).

DETECTION OF EFFLUX PUMP MEDIATED QUINOLONE RESISTANCE

Efflux pumps are considered as alternative mediators of fluoroquinolone resistance and reduced susceptibility in *E. coli*. The investigation into efflux pump mediated fluoroquinolone resistance usually involves phenotypic and genotypic methods. The two, most widely used phenotypic methods to show the presence of efflux pumps are (i) the evaluation of the changes in the minimum inhibitory concentration levels of antimicrobials by using the molecules known to inhibit the efflux pumps (EPIs) (ii) determination of intracellular accumulation and efflux of fluorescent probe ethidium bromide in the bacterial cells, in the presence and absence of EPI.

The incidence of efflux in human and veterinary isolates of resistant *E. coli* suggested that it may be an important factor in quinolone resistance. Of more than 40 putative efflux transporters in *E. coli*, mainly AcrAB-TolC overproduction has been shown to contribute to clinical fluoroquinolone resistance. Baucheron *et al.* (2012) reported high relevance of the AcrAB efflux pump in the development of quinolone resistance in *S. typhimurium* as inhibition of AcrAB operon resulted in a decrease in the MIC of enrofloxacin from 64 to 2 µg/ml and marbofloxcin from 32 to 2-4 µg/ml, against S. *typhimurium*strains. Sarah and Rania (2017) also detected a strong positive correlation of the overexpression of efflux pumps (*mdfA*and *acrA)* with resistance in *E. coli* isolates against levofloxacin. Karczmarczyk *et al.* (2011) reported up-regulation of AcrAB-TolC efflux pump in resistant *E. coli* isolates collected from food producing animals. Similarly, Toyotaka *et al. (*2013) reported that resistant *E. coli* strains isolated from human and canine fecal samples, exhibited higher expression of AcrA, greater intracellular accumulation of enrofloxacin in the presence of CCCP and a greater reduction in the MIC of enrofloxacin after addition of PAβN and suggested that AcrAB-TolC efflux pump over-expression was related to high-level fluoroquinolone resistance.

CONCLUSION

Resistance against fluoroquinolones is not simple and our understanding of the multiple mechanisms of resistance may help in fighting the spread of resistance against these drugs.This could lead to serious therapeutic crisis in conditions where humans have acquired a quinolone-resistant *E. coli* via the food chain, which may transfer resistance genes to human pathogens. According to 5th revision of the list of critically important antimicrobials published by WHO in April 2017, quinolones are classified as highest priority critically important drugs amongst the antibiotics currently used in human and animals, and thus are recommended not to be used for treatment of food producing animals unless no other drug is available (WHO, 2017). Inhibition of efflux pumps appears to be an attractive approach for improvement of the clinical efficacies of anti-bacterials that are substrates of such pumps and thus it may help in designing new combination therapeutic strategies against resistant organisms.

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Research Article

ANTIOXIDATIVE AND PROTECTIVE POTENTIAL OF *WITHANIA SOMNIFERA* IN MONOCROTOPHOS INDUCED SUBACUTE TOXICITY IN BROILERS

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ABSTRACT

The present investigation was conducted to evaluate antioxidative and protective effect of *Withania somnifera* against subacute toxicity of monocrotophos (MCP) in chickens. The study consisted of 5 groups having 12 birds in each. Monocrotophos (2 ppm in feed daily) was administered to birds of group II to V and *W. somnifera* aqueous root extracts, WRE (100, 300 and 500 mg/kg b. wt/day) were given in drinking water to birds of group III, IV and V birds respectively for 7 weeks. Group I served as control. Increase in lipid peroxidation (LPO) and decrease in the activity of antioxidant enzymes GSH and catalase in RBC, liver and brain ($P < 0.05$) were observed in the subacute toxicity of MCP. However, WRE showed marked decrease in LPO and increase in reduced glutathione and catalase activity in erythrocytes and tissues of MCP group (II) birds when compared to control. FCR, body weight and organ weights (liver, brain and kidney) were reduced in MCP exposed Group II birds while these parameters were improved in WRE treated birds. These observations suggested protective role of *W. somnifera* aqueous root extract against MCP-induced oxidative alterations and toxicity.

Key words: Monocrotophos, glutathione, catalase, *W. somnifera,* FCR

INTRODUCTION

Monocrotophos is an AChE inhibiting organophosphate compound used as acaricide and antihelmintic agent. Its indiscriminate use has caused deleterious effects in exposed birds. Medicinal herb like *W. somnifera* is a known antistress, adaptogenic and hemopoietic substance as described in Ayurvedic system of medicine (Mishra *et al.,* 2000). Glycowithanolides, sitoindosides VII- X and withaferin A are active principle that provide antioxidative potential to *W. somnifera* by increasing activities of superoxide dismutase, catalase, glutathione peroxidase (Bhattacharya *et al.,* 2000; Mishra *et al.,* 2000).

The present investigation was undertaken to evaluate protective potential of *Withania somnifera* aqueous root extract (WRE) on oxidative profile and ameliorating effect in subacute monocrotophos (MCP) toxicity (2 ppm in feed) daily in broiler chickens.

MATERIALS AND METHODS

Preparation of plant extract

Withania somnifera aqueous roots were grinded, soaked in distilled water for 24 h and filtrate was dried in incubator with fan and finally lyophilized

Experimental animals and design

In this experiment 60 day-old commercial broilers were divided into five groups (12 in each). The broilers of group II to V were treated with 2 ppm of MCP in feed daily with effect from day 7 till the end of experiment. *Withania somnifera* root extract, WRE was given @ 100, 300 and 500 mg kg^{-1} b. wt in morning drinking water daily to the birds of group III to V and thereafter till end of the experimental birds were kept in battery cage system under standard management conditions and were given feed and water ad libitum throughout the study. Birds of group I did not receive any treatment except routine feed and water served as untreated control. On 43rd day, all the experimental birds were sacrificed. Blood samples and tissues (liver, brain and kidney) were collected to evaluate antioxidative and histopathological parameters. After completion of experiments all the birds were sacrificed and buried humanely and scientifically.

Antioxidative parameters

Estimation of different oxidative stress related biochemical parameters in erythrocytes, liver and brain was carried out. Blood samples were presented for separation of erythrocyte pellet immediately after sacrifice, while liver and brain were kept at - 20ºC till further use. Absorbance of all the estimations were read, using double beam VIS and UV- VIS Spectrophotometer (UV 5704 ss, ECIL, India). Estimation of LPO, lipid peroxidation product was determined in terms of malondialdehyde (MDA) production by the method of

Rehman (1984) in tissues homogenates (liver and brain) and in RBC. Reduced glutathione was essayed in RBC by the 5, 5- dithiobis (2-nitrobenzoic acid) (DTNB) method of Prins and Loos (1969) and in tissue homogenates (liver and brain) to estimate free-SH groups using DTNB method of Sedlak and Lindsay (1968). The 10 % dilution RBC in PBS was used for estimation of catalase as per the method described by Bergmeyer (1983).

Separation of erythrocytes

The heparinized blood samples were centrifuged at 2000 rpm for 15 min. Plasma and buffy coat were removed and resulting erythrocyte pellet was washed thrice with 0.15 M NaCl. The 33 % dilution of the packed RBC was made in PBS (pH 7.4) (Yagi, 1989). This 33 % packed RBC was used for estimation of lipid peroxidation and reduced glutathione. The washed erythrocyte pellets were suspended in PBS (pH, 7.4) and kept at 4 ^{oC} till further analysis.

Preparation of tissue homogenate: Frozen liver and brain samples were partially thawed and 200 mg of each sample was weighed and taken in 2 ml of ice-cold saline. Tissue homogenates were prepared by trituration using pestle and mortar.

Histopathology

Tissues from spleen, liver, kidney, brains etc. were collected (Luna, 1968). Sections of 3-5 mm thickness were cut and stained with hematoxylin and Eosin (H&E) for observation under light microscope.

Statistical analysis

One way analysis of variance (ANOVA) was employed to compare effects of treatments on body weight, FCR and antioxidative parameters by the method of Snedecor and Cochran (1989).

RESULTS

Clinical signs

Group II birds exposed to monocrotophos (2 ppm in feed) were found to be dull, depressed with poor growth while birds of other groups did not produce any typical sign of toxicity (Table 1).

FCR (Feed conversion ratio)

There was significant decrease in FCR of MCP group (1.56 ± 0.06) as compared to control group (1.95 ± 0.12). However, WRE treated birds of group V exhibited increase in FCR (1.82 ± 0.02) as compared to group II (Table 1).

Body weight and organ weights

There was reduction in body weight gain in MCP exposed group II chickens after 2 weeks which remained depressed till end of experiment (7 weeks), as compared to control birds. Alternatively, *W. somnifera* extract treated birds showed significant increase in body weight when compared to MCP group birds (Table 1). Commercial broilers of group V exhibited significant increase in body weight after 3, 4, 6 and 7 weeks while group IV birds after 5 weeks respectively (Table 1). Significant reduction in absolute weight of liver and brain in MCP group was observed (Table 1) while weights of liver and brain were significantly increased in WRE treated group IV birds. Also, group V birds showed significant increase in absolute liver weight. There was no significant difference in weight of kidneys between treated and untreated groups and in relative weights of liver, brain and kidneys (Table 1).

Histopathological findings

The birds were sacrificed scientifically at end at the 7 weeks study. Necropsy findings exhibited generalized congestion and hemorrhages throughout the body of MCP-treated birds. On microscopic examination, liver showed local area of degeneration and necrotic changes in subacute study. Hemorrhage, vacuolar degeneration and necrosis were observed in kidneys. Brain was atrophied, hyperemic and neurons degeneration was evident. In group V birds less pathological lesions were seen.

Lipid peroxidation

Ameliorating effect of *W. somnifera* was

Table 1.
Body weight and FCR of birds of different groups after 7 weeks of MCP (2 ppm in feed) treatment (mean±S.E, n=12).

Group	I(Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE(mg/kg/day)	-	-	100	300	500	C.D	d. f	F
B. wt at 0 day(g)	31.2±0.76	27.7±1.43(11)	29.2±0.72(5)	29.7±0.85(7)	31.2±1.31(12)	2.99	4,30	1.85
After 1 wk	64.3±2.36	58.3±2.35(9)	60.0±1.67(2)	63.2±4.83(8)	62.2±3.83(6)	7.83	4,30	0.76
After 2 wks	149.2±6.9	114.1±3.56[a](23)	124.2±9.57(8)	131.7±7.3(14)	129.2±5.43(13)	19.4	4,30	3.49
After 3 wks	329±12.7	257.7±11.8[a](21)	310.7±19.1(20)	300.2±18.3(16)	315.8±13.9[b](22)	43.8	4,30	3.10
After 4 wks	568.7±21	461.5±26.5[a](18)	502.5±28.1(8)	577.7±21.6 (25)	531.7±24.0[b](15)	69.3	4,30	2.58
After 5 wks	861.7±38	735.8±13.3[a](14)	786.7±38.6(16)	839.2±28[b] 6 (14)	817.9±24.4(11)	86.2	4,30	2.59
After 6 wks	1065±46	874.2±24.7[a](17)	908.3±39.0(3)	950±40(8)	1004.2±49 [b] (14)	116	4,30	3.48
After 7 wks	1127±44	925.8±27.3[a](17)	994.2±36.1(7)	1002.5±32 (8)	1037.5±32[b] (12)	99.7	4,30	4.37
FCR	1.95±0.12	1.56±0.06[a](20)	1.71±0.10(9)	1.79±0.08(14)	1.82±0.02 [b](16)	0.24	4,25	2.94

[a] = p< 0.05 as compared to untreated control in the same row., [b] = p< 0.05 as compared to MCP group in the same row.
Values in parenthesis are % change as compared to control group

Table 2.
Effect of aqueous root extract of *W. somnifera* (WRE) on antioxidative profile in broiler chickens after 7 weeks of monocrotophos (MCP) treatment, (Mean ± S.E., n=6).

Group	I (Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE (mg/kg/day)	-	-	100	300	500	C.D	d.f	F
LPO (nM.MDA/ ml RBC)	2.10 ± 0.96	4.65± 0.69[a] (121)	4.22± 0.72 (9)	2.36 ± 0.40[b] (49)	2.18 ± 0.57[b] (53)	2.07	4,20	3.08
LPO (nM.MDA/g Liver)	2.65±0.87	6.14±0.48[a] (131)	4.43±0.78 (27)	3.44±0.68[b] (43)	2.70±1.16[b] (56)	2.44	4,20	3.12
LPO (nM.MDA/g Brain)	2.08±0.17	4.15±0.3[a] (86)	3.85±0.27 (19)	3.76±0.27 (27)	2.75±0.34[b] (45)	0.83	4,20	9.82
GSH (mM/ml RBC)	73.7±3.80	36.8±10.7[a] (50)	25.5±13.9 (30)	20.6±18.6 (43)	57.2±10.9 (55)	36.5	4,25	3.14
GSH (mM/mg Liver)	52.1±2.8	24.1±1.78[a] (54)	41.7±3.79[b] (73)	25.3±2.15 (5)	28.2±3.04 (17)	8.12	4,25	18.9
GSH (mM/mg Brain)	42.4±6.65	19.3±4.61[a] (54)	33.9± 9.77 (75)	21.95±3.6 (13)	41.8±4.72[b] (116)	18.5	4,20	2.98
Catalase* (RBC)	346.8±47.2	169.5±23[a] (51)	229.3±9.6 (35)	218.8±41.1 (29)	290.8±28[b] (71)	90.1	4,20	5.06

[a] = p< 0.05 as compared to untreated control in the same row., [b] = p< 0.05 as compared to MCP group in the same row.
* mM H_2O_2 utilized / min/ mg Hb, Values in parenthesis are % change as compared to control group.

assessed against subacute toxicity of MCP. The status of lipid peroxidation, reduced glutathione in (RBC, liver and brain) and catalase enzyme activity (in RBC) were analyzed and are summarized in Table 2. Lipid peroxidation in RBC of MCP group birds was found to be significantly increased (4.65 ± 0.69 nM. MDA/ ml RBC). Lipid peroxidation product in group IV and V birds were significantly reduced (Table 2). Significant increase in lipid peroxidation product in tissues (liver and brain) of MCP exposed group II birds was found compared to control birds, respectively. However, significant reduction in the level of LPO in liver was observed in group IV and V birds and in brain of WRE-treated group V birds (Table 2).

Reduced glutathione (GSH)

Compared to control birds, the level of reduced glutathione was significantly decreased in RBC, liver and brain of MCP group birds (Table 2). No significant increase in GSH level in RBC was not observed in any of the treated group. However, there was significant increase in the level of GSH was observed in liver of WRE-treated group III birds and in brain of group V birds respectively.

Catalase

A significant decrease in the activity of catalase was analysed in chickens of MCP group. Conversely, birds of group V showed marked increase in the activity of catalase (290.8 ± 28.1 mM H_2O_2 utilized / min/ mg Hb) in comparison to MCP group II.

DISCUSSION

Body weight and FCR

In present study, there was no overt signs of toxicity of MCP in broiler chickens of any treated group though the birds of group II remained dull and depressed throughout the study period. In present study, body weight and FCR were significantly decreased in birds having subacute exposure of MCP. Progressive retardation in growth due to anorexia and systemic degenerative and necrotic changes may account for the reduced body weight and FCR in birds of MCP group. Chronic exposure of chicks to low dose of MCP caused deleterious effect on metabolism and immune system of birds (Garg *et al.,* 2004). Ashwagandha root extract had positive effect on body weight in mice (Ziauddin *et al.,* 1996) and broiler birds (Samarth *et al.,* 2002). Our observations are in conjunction with these findings. WRE-treated birds showed significant & dose-dependent increase in body weight as compared to MCP group 3 weeks onwards till the end of the experiment. The value of FCR was also significantly increased by WRE in group V birds. Thus, WRE counteracted the MCP-induced decrease in body weight and FCR.

Organ weights

Significant reduction in the absolute weights of liver and brain in MCP group birds was observed as compared to control. Reduction in organ weight may be attributed to atrophy and degeneration of organs and alternation in protein metabolism. This is supported by histopathological changes in organs. WRE-treated birds showed significant increase in the weights of liver and brain as compared to MCP group. Prolonged medication of *W. somnifera* shows positive effect on weight gain, feed consumption, general resistance and liver weight in man and animals (Archana and Namasivayam, 1999; Rao *et al.,* 1999). These findings reinforce the hepatoprotective (Bhattacharya *et al.,* 2000) and neuroprotective effects of *W. somnifera* (Parihar *et al.,* 2004).

Oxidative profile

Reactive oxygen species (ROS) viz. hydroxyl radical (HO^-), superoxide anion radical (0_2^-) and hydrogen peroxide (HOOH) are normally formed during β-oxidation of fat in peroxisome, auto-oxidation of amino acids, hemoglobin and catecholamines and mitochondria electron transport chain. Cells are said to be in oxidative stress when generation of reactive oxygen species (ROS) exceeds the cells capacity to scavenge ROS. Various stress factors like drugs, toxins, tissue injury and heavy physical work may accelerate overproduction of ROS. As per Sakaguchi and coworkers reports (1981), ROS is known to increase LPO by acting as pyrogen and stimulating formation of free radicals. These free radicals have nociceptive effect on membrane lipids (Formann, 1989).

ROS-mediated lipid peroxidation damages structural integrity of cell membrane and results in disruption of lipid, protein and DNA via formation of aldehydes. These oxidative damages can be prevented by antioxidant defense mechanism in form of antioxidant enzymes catalase, glutathione reductase, lipid peroxidase, etc. Catalase causes reductive degeneration of HOOH to form water and oxygen. While GSH protects against ROS-mediated oxidative damage. The balance between oxidation of GSH to glutathione disulfide (GSSG) and the rapid reduction of GSSG to GSH by GSH glutathione reductase contributes to the maintenance of cellular homeostatic GSH:GSSG ratio of about 300:1 (Alpert and Gilbert, 1985). Deficient functioning of catalase and reduced glutathione may lead to accumulation of toxic oxidative free radicals and consequently degenerative changes (Maxwell, 1995) via peroxidation of membrane lipids (Balin and Allen, 1986).

As per Tang (2001) reports, the level of cell membrane lipid peroxidation product malondialdehyde (MDA) and the rate of cellular electrolyte leakage were found to be increased in MCP-treated marine microalgae. Significant depletion in the level of reduced glutathione and inhibition in the activities of glutathione-S-transferase and UDP-glucoronyl transferase in rats was showed by two analogs of monocrotophos-RPR-II and RPR-V (Mahboob and Siddiqui, 2001). Singh and coworkers (2006) reported increased activates of glutathione-S-transferase and glutathione reductase along with decreased glutathione content in RBC in MCP-treated rats. Also, Rao (2006) observed depletion of glutathione level in plasma, liver muscle, brain, kidney and gill of euryhaline fish exposed to MCP @ 1.15 mg/l for 30 days.

The object of present investigation was to demonstrate subacute exposure of broiler birds to MCP resulting in increased lipid peroxidation (LPO) in erythrocytes, liver and brain as evidenced by the increased production of malondialdehyde (MDA) in group II birds. Also, there was decrease in activity of antioxidant enzyme catalase and level of reduced glutathione (GSH) in RBC. Formation of free radicals caused oxidative cellular injury in MCP-treated birds as evidenced by elevation of LPO in erythrocytes. It might be due to peroxidation of unsaturated fatty acids in plasma membrane phospholipids of RBC. Thus increased LPO in RBC in suggestive of progressive increase in cellular deformity, increase in membrane permeability and rigidity and disruption of structural and functional integrity of cell organelles (Corry *et al.,* 1970). Increased production of MDA in liver and brain of MCP-treated birds signified generation of ROS in these tissues through oxidative damage to cell membrane. While LPO was increased there was significant reduction in antioxidant marker enzymes catalase in RBC and GSH in RBC, liver and brain. Inhibition of glutathione reductase activity leading to accumulation of GSSG by preventing reduction of GSSG to GSH suggested that consumption of GSH may not be compensated by glutathione reductase enzyme resulting in reduced glutathione content.

In present study ashwagandha root extract showed significant decrease in lipid peroxidation and increase in GSH level and catalase activity in erythrocytes and tissues of MCP-treated birds. *W. somnifera* root contains active glycowithanolides which caused marked increase in antioxidative potential by increasing the activities of superoxide, dismutase, catalase and glutathione peroxidase (Bhattacharya *et al.,* 1997). Sitoindoside –VII and withaferin A are active principles in *W. somnifera* root extract which caused significant increase in oxidative free radical scavenging enzymes along with decrease in lipid peroxidation (Panda and Kar, 1998a). The inhibition of lipid peroxidation by *W. somnifera* root extract was also reported by earlier workers in cadmium-, copper- and LPS-intoxicated animals (Dhuley, 1998a; Gupta and Kaur, 2002). Investigation by Bhattacharya and coworkers (2000) revealed that the equimolar concentration of sitoindoside VII-X and withaferin A provide dose-dependent protection against iron overload-induced oxidative damage. These findings are also in confirmation with our results, in which there was dose dependent increase in antioxidant enzyme catalase, level of GSH and decrease in lipid peroxidation product.

Enhancement in catalase activity and GSH level while reduction of lipid peroxidation were observed in birds following treatment with WRE. Hence, MCP–mediated oxidative stress was reduced by *Withania somnifera.*

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Research Article

EFFECT OF ETHANOLIC LEAF EXTRACT OF *AEGLE MARMELOS (L.)* CORREA ON GENTAMICIN INDUCED NEPHROTOXICITY IN RATS

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ABSTRACT

The aim of the present study was to evaluate the protective effect of ethanolic leaf extract of *Aegle marmelos* Correa. on gentamicin induced nephrotoxicity in Wistar rats. The rats were divided into 4 groups of 12 each, Group A serve as Positive control and Group E vehicle control, while Group B, C, D were orally treated with Gentamicin (40mg/kg), Gentamicinplus Ethanolic leaf extract of Aegle Marmelos @ 250mg/kg and Gentamicn plus Taurine @ 1000mg/kg respectively for 14days.Gentamicin administration cause elevated Serum alanine aminotransferase(ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN),serum creatinine and uric acid values. Administration of ethanolic leaf extract of Aegle marmelos with gentamicin significantly decreased the level of Serum alanine aminotransferase(ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN),serum creatinine and uric acid values. There were significant decreased in body weight and increased in organ weight. The result indicates that *Aegle marmelos* ameliorate the gentamicin induced renal damage in rats.

Key words: *Aegle marmelos* Correa, Gentamicin, Nephrotoxicity, Wistar rats Taurine.

INTRODUCTION

Gentamicin (GM) is an aminoglycoside antibiotic commonly used in treating life-threatening gram-negative bacterial infections (Ali, 1995). However, 30% of the patients treated with GM for more than 7 days show some signs of nephrotoxicity and serious complications resulting from GM-induced nephrotoxicity which is a limiting factor for its clinical usage (Mathew, 1992). GM is not metabolized in the body but is essentially eliminated by glomerular filtration and partially reabsorbed by proximal tubular cells (Black *et al.,* 1963; Silverblatt*et al.,* 1979). Gentamicin-induced kidney damage is characterized by tubular necrosis, primarily localized to the proximal tubule. This might be explained by an increased kidney uptake of the antibiotic, mainly at the proximal tubular level. Gentamicin induces superoxide stress anions and hydroxyl radical production in mitochondria and acts as iron chelator. Moreover gentamicin enhances the generation of reactive oxygen species which could be important mediator of nephrotoxicity. It has been shown that chelator and antioxidant property reduce gentamicin induced oxidative stress in--*vitro* and in-*vivo* (Sha & Schacht, 2000).

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Medicinal plants have curative properties due to the presence of various complex chemical substances. Ancient literature has prescribed various plants for the cure of kidney disease. Co-administration of various medicinal plants possessing nephroprotective activity along with different nephrotoxic agents may attenuate its toxicity. *Aegle marmelos (L.)* Correa belongs to family Rutaceae, commonly known as Bael (Hindi), and Golden apple (English). The plant *Aegle marmelos* contains number of phytochemicals like. Aegeline, agelinine, rutin, sterol, β sitosterol, glucoside, marmesinine, tannins, flavonoids and quercetin and volatile oils triterpenoids, amino acids. The different parts of Bel are used for various therapeutic purposes such as for the treatment of diabetes, Asthma, Anaemia, antidote to snake venom, fever, dysentery, chronic diarrhoea, heart disorders, jaundice (Kirtikar and Basu, 1993). Leaf extract of the Bel was found to be a potential antioxidant drug, which improves antioxidant property of glutathione, glutathione peroxidase, glutathione reductase, superoxide dismulase (SOD) and catalase (Kuttan and Sabu, 2004). Aqueous leaf extract of *Aegle Marmelos (L.)* Correa. was found to be effective in preventing Gentamicin (GM)-induced nephrotoxicity in Wistar rats (Kore *et al.,* 2011). Therefore, the present study has been undertaken to evaluate protective effect of Aegle Marmelos on gentamicin induced nephrotoxicity in wistar rats at KNP College of Veteriary science, ShirvalDist Satara, Maharashtra.

MATERIALS AND METHODS

Preparation of extract

Aegle Marmelos Correa. leaves were procured from local area of Nanded &Shirwal village. They were dried under shade and then pulverized into fine powder by using mixer grinder. Ethanolic extract of leaves of *Aegle marmelos* was prepared and extractability

percentage was determined as per the method suggested by Rosenthaler (1930). 50 grams of leaves powder of *Aegle marmelos* (L).Correa was placed in thimble made up of thick filter paper which was loaded into main chamber of the Soxhlet extraction apparatus. After extraction, the phytochemical analysis of all the extracts was carried out to find out the presence of active principles as per Rosenthaler (1930).

Experimental design

60 Wistar rats of either sex were divided randomly into 6 groups with 12 animals in each. The rats were housed in clean polypropylene cages, under controlled environmental conditions (25+-2^{0}C) and 12:12 light: dark cycle with sterilized dried clean, autoclaved paddy husk was used as bedding material, which was changed on alternate days. The rats were maintained on standard died with clean purified aqua guard filtered drinking water *ad libitum* throughout the experimental period. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Group A served as a negative control while Group B rats were injected with gentamicin sulphate @ 40 mg per kg body weight IP daily for 14 days. Group C rats were given ethanolic leaves extract *Aegle marmelos* @ 250 mg per kg body weight orally as a preventive regimen along with the injection of gentamicin sulphate @ 40 mg per kg body weight IP for the period of 14 days starting of days post treatment. Group D rats were injected with gentamicin sulphate @ 40 mg per kg body weight IP and Taurine @ 1000 mg per kg body weight orally dissolved in distilled water for 14 days. Group E rats were given Tween 20 with drinking water orally for 14 days (vehicle control). After the administration of last dose, all the rats from entire groups were fasted for 24 hours and blood samples from all the animals in two aliquots were collected (one in anticoagulant added vial for hematological studies and other in anticoagulant free vial for biochemical estimations) at 0, 14 and 21 days post treatment.

Histopathological Examination

The tissue samples viz.kidney and liver from all animals of each group were collected in 10 % Neutral Buffered Formalin and processed by routine paraffin embedding method. The tissue sections of 3 to 5 microns size were stained by routine Haematoxylin and Eosin staining as suggested by Chauhan (1995)

Statistical analysis

The data collected for various parameters were statistically analyzed by using analysis of variance (ANOVA) using WASP 2 computer software. All the values in the text are expressed as mean +-SE.

RESULTS AND DISCUSSION

Qualitative phytochemical tests of different of ethanolic leaf extract of *A. marmelos* Correa revealed presence of flavonoids by Ferric chloride & Shinoda test, saponins by Foam test, gum and mucilage by alcohol 95% test, fixed oil and fats by spot test and saponification test, carbohydrate and glycosides by Fehling's and Benedict's test, phenolic compound and tannin by Lead acetate and ferric chloride tests, alkaloids by Dragendorff's test, Similar observations were reported by Pandey and Mishra (2011). All the rats were observed daily thrice for clinical signs and abnormal behavior. It was observed that the animals of group A, C, E and F did not show any symptoms throughout the experimental periods. In group B, both male and female rats showed varying degree of clinical signs including restlessness,

Table 1:

Effect of relative kidney and livr weight of rats on administration of *A. marmelos* leaf extract

Group	Treatment	Relative Kidney Weight (%)	Relative liver weight (%)
A	Control	0.321±0.009^c	3.323±0.053^b
B	Gentamicin sulphate @ 40 mg/kg body weight	0.506±0.012^c	5.213±0.179^a
C	Gentamicin sulphate @ 40 mg/kg body weight + *A.marmelos*Corr.leaves extract @250 mg/kg body weight	0.371±0.0019^{bc}	3.625±0.179^a
E	Gentamicin sulphate @ 40 mg/kg body weight + Taurine @1000mg/kg body weight	0.377±0.026^b	3.631±0.206^b
F	Vehicle control	0.323±0.0003^{bc}	3.361±0.069^b

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

Table 2:

Evaluation of biochemical parameters using *Aegle marmelos* Extract in rats for 14 days.

Parameter	Group I	Group II	Group III	Group IV
BUN (mg/kg)	18.08 ±0.12 $^{b\ m}$	24.83±0.31	18.18±0.41^{bc}	18.02±0.14^{bc}
Serum Creatinine (mg/kg)	0.59±0.02 ^{b}	1.33±0.03 $^{a\ m}$	0.62±0.02	0.621±0.02^b
AST/SGOT (IU/L)	62.75±0.92 $^{b\ m}$	110.05±3.06 $^{b\ m}$	63.35±1.719^b	62.81±0.83^b
ALT/SGPT (IU/L)	46.42±1.87^c	47.62±1.87	44.59±1.91^c	45.09±1.53^b

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

depression and intermittent diarrhea during the initial period of study and hair loss reduced feed and water intake at the at the end of experiment. Group D animals showed similar behaviour changes as in group B for initial 14 days but these symptoms gradually disappeared by 21 days i.e. at the end of the of experiment. Similar observations were reported by Khalid *et al.* (2003) sluggish behaviour, weak and ill looked animal treated with gentamicin @ 40 mg/kg body weight. These behavioural changes could be due to toxic effect of gentamicin. There was significant increase in relative kidney and liver weights were in group B could be attributed to degenerative necrotic and inflammatory swelling of these organs caused by toxic effect of gentamicin. Whereas Taurine and ethanolic leaf extract of *A. marmelos* Correa 250mg/kg body weight significantly improved the damage to these organs by gentamicin.

On 14th day BUN values of rats from Group A,B,C, D & E were.18.32±0.12, 24.83±0.31,18.18±0.41, 18.02±0.14 and 17.96±0.12 respectively. The BUN levels of the rats from treatment groups differ significantly ($P<0.05$) on 14th day. Significantly ($P<0.05$) higher BUN value was observed in Group B (24.83±0.31) as compared to other groups on 14th day.

The results of study indicated the increase in BUN values by 25% in Group B on 14th day than zero day. Increase in serum BUN level in rats treated with gentamicin suggested kidney damage.The results of study were in agreement with Annie et al (2005), Badwai *et al.* (2012). The results of the study indicated that, the rats administered with gentamicin @ 40 mg/kg body weight and *A.marmelos* leaf extract @ 250 mg/kg body weight did not alter the BUN values significantly which was observed in the rats treated with gentamicin alone.This might be due to protective effect of *A. marmelos* leaf extract in gentamicin induced nephrotoxic rats at early stage. Statistically significant ($P<0.05$) increase in creatinine value was observed in Group B (1.33±0.03) than other groups on 14th day. The results of the study showed the increase in creatinine values by 57.14 per cent in Group B on 14th day compared to its zero day. The results were in agreement with Annie *et al. (*2005) and Badwai *et al.* (2012). There was no significant difference in creatinine values of rats in Group A (0.59± 0.02), Group F (0.62±0.02) and Group E (0.62± 0.02) on 14th days. The values in group A, E & F .were lower than that of Group B (1.33±0.03) on 14th day.

The results of present study indicate that ,the administration of *A. marmelos* leaf extract @ 250 mg/kg body weight did not alter the creatinine level which was observed in rats administration with gentamicin @ 40 mg/kg body weight on 14th days, Similar results were recorded by Kore *et al.* (2011) recorded that aqueous extract of leaf of *A. marmelos* on rats showed significant decreased creatinine value (1.281±0.048),0.82±0.02 and 0.64±0.01) respectively compared to gentamicin group.

The results of the study indicated the increase in AST values by 42.72 per cent in Group B on 14th day compared to zero day. The results of the study were in accordance with the findings of Abdel Gayoum *et al.* (1994) reported that highest dose of gentamicin 80 mg/kg significantly lowered alanine transaminase and increased serum creatinine and serum lactate concentration with tubular necrosis compared to control group.There was no significant ($P<0.05$) difference in the AST values of the rats in Group A, C, and F. The results indicated that, rats administered with only gentamicin @ 40 mg/kg body weight showed increase in AST values while the administration of *A.marmelos*leaf extract @ 250 mg/kg body weight and gentamicin @ 40 mg/kg body weight in rats did not alter the AST value on 14th day. On 14th day, the AST values of the rats in group A, B, C, E and F were 46.421±1.876, 47.623±1.90, 44.590±1.910, 45.093±1.538, and 43.818±1.881 respectively. Significantly ($P<0.05$) increased AST value was observed in Group B (47.623±1.90) than control Group A (28.99 ± 0.945).

The results of the study indicated the increase in ALT values by 34.25 per cent in Group B on 14th day compared to zero day,than control group A (46.42±1.87). The results of the study indicated the increase in ALT values by 11.76 per cent in Group B on 14th day compared to zero day. The results were in agreement with the findings El-Ghany*et al* (2012) they reported that gentamicin treated group showed significant increased in AST,ALT and ALP ($P<0.001$) creatinine ,urea and uric acid and significant decreased and significant decreased total protein and globulin. Histopathological observation of liver showed derangement of hepatic cords with granular changes, cellular swelling of hepatocytes, congestion of central and portal blood vessel with sinusoidal congestion, focal and necrotic degenerative changes in few sections in gentamicin treated animals. The *A. marmeols* leaf extract and taurine partially reversed histopathological changes in liver. These restorative changes in liver. These restorative changes of hepatocytes might be due to *A. marmelos* and taurine therapy given during the experimental period. The mechanism involved in protective effects might be due to antioxidant activity of phytoconstituents of *A. marmelos* correa leaf extract which may act as free radical scavengers that restored gentamicin induced oxidative stress in animals. It is evident from present work that gentamicin cause tubular necrosis, increase in serum urea and serum creatinine.

Qualitative analysis for phytochemicals in

aqueous leaves extract of *Aegle marmelos* correa showed presence of alkaloids, carbohydrate, phenolic compound and saponins, tannin, fixed oils and fats.Gentamicin induced nephrotoxicity in Wistar rats is characterized by decrease in body weight, Hb, TEC, TLC and increased kidneys and liver weight, BUN, AST, ALT and serum creatinine.Histologically, gentamicin induced nephrotoxicity in rats is characterized by renal damage.Preventive dose of *A.marmelos* corr.leaves extract provided significant protection and improvement against gentamicin induced nephrotoxicity than Taurine. Preventive and curative doses of *Aegle marmelos* leaves extract had really beneficial nephroprotective effect in rats. However detailed studies are needed to confirm and study the mechanism of action of *A. marmelos* corr. in amelioration of nephropathy and isolation and identification of nephroprotective fraction of *A. marmelos* leaf extract on molecular basis.

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Research Article

DISPOSITION KINETICS OF CEFTIZOXIME FOLLOWING SINGLE DOSE INTRAMUSCULAR ADMINISTRATION IN SHEEP

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ABSTRACT

Ceftizoxime is a third generation broad spectrum antimicrobial drug, widely used in veterinary medicine. The present study was planned to investigate pharmacokinetics of ceftizoxime in sheep (n = 6) at the dose rate of 10 mg kg^{-1} body weight following single intramuscular administration. Plasma was separated from periodical blood samples drawn after drug administration. Drug concentration in plasma was determined using Ultra High Performance Liquid Chromatography (UHPLC) with UV detector. The ceftizoxime plasma concentrations versus time data after intramuscular administration was described by non-compartment model of pharmacokinetics with the use of "PK Solver Software". The LOQ of ceftizoxime was found to be 0.1 µg ml^{-1} in plasma. Following single dose intramuscular administration of ceftizoxime (10 mg kg^{-1} b. wt.), therapeutically effective concentration of ceftizoxime as 0.34 ± 0.03 µg ml^{-1} persisted in plasma up to 36 h. The average mean values of elimination rate constant, half-life, area under curve, mean residence time and total body clearance were found as 0.07 h^{-1}, 9.97 h ,152.32 µg h ml^{-1},9.48 h and 0.07 L h^{-1} kg^{-1}, respectively.

Key Words: Pharmacokinetics, ceftizoxime, intramuscular, sheep

INTRODUCTION

Antimicrobial drugs are the most frequently prescribed and administered drugs in veterinary medicine (Dowling, 1996). Cephalosporins has played a major beneficial role in allopathy considering its characteristics like quick and wide distribution, good therapeutic ratio, and excellent tissue penetration. Ceftizoxime is a third generation cephalosporin having high bactericidal activity against a wide range of Gram-positive and Gram-negative microorganisms including *Streptococci, Staphylococci, Proteus, Bacillus, Klebsiella, Clostridium, Salmonella* and *Shigella spp*. (Mandell, 1979). It is commonly used for the treatment of the infections of respiratory tract, urogenital tract, skin, soft tissues, bones and joints. Ceftizoxime has certain pharmacological and clinical advantages over other cephalosporin. It has better activity against anaerobes, broader spectrum of activity against Gram negative bacteria, penetrates the cerebrospinal fluid in sufficient concentration due to greater lipid solubility, and is resistant to hydrolysis by β-lactamase (Mandell and Sande, 1991). Ceftizoxime is not metabolized in the body and is excreted unchanged predominantly by glomerular filtration (Facca *et al.,* 1998). The acceptance of ceftizoxime in clinical veterinary medicine mainly depends on designing and planning pharmacokinetic studies in various animal species and constructing the dosage regimen based on it. Previously, pharmacokinetic studies of ceftizoxime were carried out in various species *viz*., mice, rats, dogs and monkeys (Murakawa *et al.,* 1986), sheep (Rule *et al.,* 2000), goats (Shaktidevan *et al.,* 2005., Karmakar *et al.,* 2011) and, cross breed calves (Singh *et al.,* 2008). It is inevitable to study pharmacokinetics of drugs in animals where they are rearing as to measure influence of environment and breed physiology on drug effectiveness in body. Hence, on the ground of paucity in such research work in Indian breed of sheep, the present study was designed to determine the pharmacokinetic data of ceftizoxime in Patanwadi sheep (*Ovis aries*) after single dose intramuscular administration, under tropical environment.

MATERIALS AND METHODS

Animals

Six female healthy sheep of Patanwadi breed having body weight between 25-35 kg and age of 2-4 years were selected for the study. The sheep were housed, in clean experimental pens, two weeks prior to experiment for acclimatization. All essential and standard managemental practices were followed to keep the sheep free from any stress. This study was prior recommended by Institutional Animal Ethics Committee (IAEC), College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar.

Experimental design and sample collection

Injectable ceftizoxime powder formulation was obtained from Intas Pharmaceuticals Ltd., Ashram road, Ahmedabad. Drug was administered intramuscularly at the dose rate of 10 mg kg^{-1} to each of six sheep. 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), 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h and 48 h, post intramuscular administration of drug. Plasma was separated after centrifugation (10 min, 5000 RPM, 4^{0}C) of blood samples and stored at –

4^{0}C until analyzed.

Sample preparation for ceftizoxime assay

Ceftizoxime was extracted from plasma using ice-cold acetonitrile. Exactly, 300 µl of plasma sample was mixed with 300 µl ice-cold acetonitrile. After thorough mixing with vortex mixture (1 minute), samples were centrifuged (10 min) at 10,000 RPM at 4^{0}C using refrigerated centrifuge machine. Supernatant thus obtained was collected and 20 µl was injected into UHPLC machine.

Chromatographic conditions and validation

UHPLC apparatus (Thermo Fisher, Germany) consisting of UV detector (Dionex ultimate 3000), Gradient Solvent Delivery Pump (Dionex ultimate 3000) and manual injector was used for this study. Chromatographic separation was performed by using reverse phase C_{18} column (ODS; 25 cm x 4.6 mm ID, 4.5 µ) at room temperature. The data integration was performed by "Chromeleon" software version 6.8. The mobile phase was a mixture of 286 µl glacial acetic acid in 250 ml HPLC water and acetonitrile (60:40). Mobile phase was filtered through 0.45 µm filter paper by using vacuum pump and degassed using sonicator and pumped into column at a flow rate of 0.8 ml min^{-1} at ambient temperature. The effluent was monitored at 260 nm wavelength. The retention time of ceftizoxime in plsama samples was 3.2 min.

Calibration curve was prepared by adding known amount of ceftizoxime to blank unfortified (drug-free) pooled sheep plasma in the concentration range from 0.1 to 80 µg ml^{-1}, which were processed exactly as done for samples, described above. The assay was responsive, reproducible and linearity was observed from 0.1 to 80 µg ml^{-1} with mean correlation coefficient (R^2) of 0.9976. The mean recovery percentage of ceftizoxime in plasma was 98.41 % at 80 µg ml^{-1}. Intraday and interday precision and accuracy were calculated and at all concentrations, the value of the RSD was less than 10 %.

Pharmacokinetic analysis

The plasma concentration - time curves of individual sheep were analyzed for obtaining pharmacokinetic parameters with the software 'PK Solver', a freely available menu-driven add-in program for Microsoft Excel written in Visual Basic for Application (VBA) in solving basic problems in pharmacokinetic (Zhang *et al.,* 2010).

RESULTS

The graphical representation of mean plasma concentration of ceftizoxime against time, after its single dose IM administration (10 mg kg^{-1} b. wt.), is presented in Figure-1 as a semi-logarithmic plot. Following IM administration, the peak plasma ceftizoxime concentration was found as 30.03 ± 1.27 µg ml^{-1} at 0.25 h (15 min). Thereafter, plasma level of ceftizoxime diminished gradually (0.34 ± 0.03 µg ml^{-1} at 36 h) and was not detectable post 48 h of drug administration.

Various pharmacokinetic parameters calculated from plasma concentrations of ceftizoxime after its single dose IM administration (10 mg.kg^{-1} b. wt.) in female Patanwadi sheep are shown in Table-1. The mean elimination rate constant (β) was 0.07 h^{-1} having range of 0.07 to 0.08 h^{-1} with corresponding mean value of elimination half-life as 9.97 h having the range of 9.50 to 10.20 h. The mean value of C_{max} was 30.03 µg ml^{-1} ranging from 26.69 to 33.47 µg ml^{-1}. The average values for area under curve (AUC) and AUMC of plasma ceftizoxime concentration versus time were 152.32 µg.h.ml^{-1} and 1447.38 µg h^2 ml^{-1}, respectively. The average value of mean residence time (MRT) was 9.48 h showing a wide range of 8.82 to 10.17 h. The mean values of $V_{d(area)}$ was 0.70 L kg^{-1}, respectively. The mean value for total body clearance (Cl_B) was 0.07 L h^{-1} kg^{-1}.

DISCUSSION

In present study, the plasma levels of ceftizoxime were estimated by UHPLC system. Following single dose IM administration of ceftizoxime

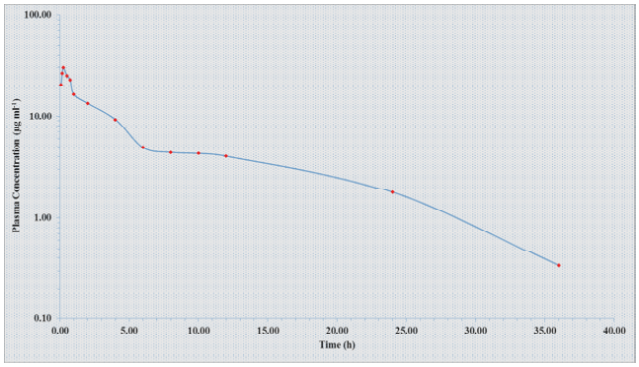

Fig. 1:
Semi logarithmic plot of mean ceftizoxime concentrations in plasma versus time following single dose IM administration (10 mg.kg^{-1} b. wt.) in Patanwadi sheep (n=6).

Table 1:
Pharmacokinetic parameters of ceftizoxime in plasma after single dose IM administration (10 mg.kg^{-1} b. wt.) in female Patanwadi sheep (n=6).

Pharmacokinetic parameters	Unit	Values (IM route)
β	h^{-1}	0.07 ± 0.00
C_{max}	µg ml^{-1}	30.03 ± 1.27
$t_{½β}$	h	9.97 ± 0.15
AUC	µg h ml^{-1}	152.32 ± 2.37
AUMC	µg h^2 ml^{-1}	1447.38 ± 65.60
Vd_{area}	L kg^{-1}	0.70 ± 0.03
Cl_B	ml h^{-1} kg^{-1}	0.07 ± 0.00
MRT	h	9.48 ± 0.29
F	%	132.12 ± 18.42

β, Elimination rate constant; C_{max}, maximum drug concentration; $t_{½β}$, elimination half life; AUC, area under plasma drug concentration-time curve; AUMC, area under first moment of curve; Vd_{area}, apparent volume of distribution; Cl_B, total body clearance; MRT, mean residence time; F, Bioavailability.

at the rate of 10 mg kg^{-1} b. wt., the mean peak plasma level found at 0.25 h (15 min) was 30.03 µg ml^{-1}. Similar peak plasma level (25.54 and 24.90 µg ml^{-1}) of ceftizoxime were reported in goat and cross breed calves following IM administration (Bhatiya, 2015, Shakthidevan *et al.,* 2005). For a successful therapeutic effect, the plasma concentrations of an antimicrobial agent should not fall below MIC during the course of treatment. In present study, the plasma ceftizoxime level at 36 h was detected as 0.34 µg ml^{-1} which was above MIC range of 0.2 µg.ml^{-1}.

In present study, elimination rate constant (β) observed was 0.07 h^{-1} with corresponding elimination half life ($t_{1/2\beta}$) of 9.97 ± 0.15 h, which demonstrate long elimination phase of ceftizoxime kinetics in sheep. However, higher mean $t_{1/2\beta}$ value of 15.71 h was reported by Bhatiya (2015) in goats following ceftizoxime IM administration. In other species like cross breed calves (Shakthidevan *et al.,* 2005) lower values of ceftizoxime $t_{1/2\beta}$ was reported as 1.64 h. In present study, the mean apparent volume of distribution calculated by area method (Vd_{area}) was 0.70 L kg^{-1} which indicated wider distribution of the ceftizoxime in sheep body. The mean value of Vd_{area} were reported lower in cross breed calves as 0.58 L kg^{-1} (Shakthidevan *et al.,* 2005) and higher in goat 0.77 L kg^{-1} (Bhatiya, 2015). In present study, mean values of AUC was found as 152.32 µg h ml^{-1}. In sheep (Rule *et al.,* 2000), cross breed calves (Shakthidevan *et al.,* 2005), goat (Bhatiya, 2015) lower values of drug AUC were noticed as 65.40, 39.20 and 98.73 µg h ml^{-1}, respectively. Longer value of mean resident time (MRT) for ceftizoxime observed as 9.48 h, indicated long persistence of this drug in sheep body. Comparatively, higher values of MRT was reported in goats as 13.00 h (Bhatiya, 2015). Total body clearance (Cl_B) was found as 0.07 L h^{-1} kg^{-1} after IM administration of ceftizoxime (10 mg kg^{-1} b. wt.) in present study, whereas, higher values of Cl_B in goat as 0.10 and 0.25 L h^{-1} kg^{-1} were observed by Bhatiya, 2015 and Shakthidevan *et al.,* 2005, respectively.

The longer persistence of ceftizoxime in blood, higher volume of distribution and longer half life provides clue to brighter clinical prospectus of ceftizoxime in the treatment of diseases caused by susceptible microorganisms in sheep. These favourable pharmacokinetics parameters combined with broad spectrum of antimicrobial activity and lower MIC values of ceftizoxime for pathogens of veterinary importance are added advantages from clinical point of view.

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Research Article

PHARMACOKINETICS OF OXYTETRACYCLINE FOLLOWING SINGLE DOSE ORAL ADMINISTRATION IN POULTRY

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ABSTRACT

The present study was undertaken to investigate the pharmacokinetics of oxytetracycline @ 50 mg.kg^{-1} in poultry birds following single dose oral administration. The concentration of oxytetracycline in poultry plasma was analysed by HPLC. The initial peak plasma concentration of 1.35 µg.ml^{-1} of oxytetracycline was detected in poultry following single dose oral administration. The volume of distribution (V_F), clearance of drug (CL_F), mean area under curve (AUC), absorption half-life (K_{01}_HL) and elimination half-life (K_{10}_HL) calculated was 16103.43±1735.73 ml.kg^{-1}, 4206.2±278.282 ml.h^{-1}.kg^{-1}, 12.156±0.819 h. µg.ml^{-1}, 2.178±0.263h and 2.737±0.402h respectively, following single dose oral administration of oxytetracycline. The therapeutic concentration (0.5 µg.ml^{-1}) of oxytetracycline was maintained upto 8hrs following single (50mg/kg) oral administration. Based on the pharmacokinetic data, an oral dosage regimen of oxytetracycline with a priming dose of 73.0 mg.kg ^{1} followed by a maintenance dose of 65.0 mg.kg ^{1} at 8 h interval is recommended.

Key words: oxytetracycline, oral, broilers, pharmacokinetics.

INTRODUCTION

Oxytetracycline (OTC) is a member of the tetracycline group of antimicrobial agents used in the prophylaxis and treatment of many infectious diseases in humans and animals and recognised for high antibacterial efficacy and low toxicity (Chopra and Roberts, 2001). The pharmacokinetics of OTC has been reported for many species (Papich and Riviere, 2010) but fewer reports in broiler chickens by different routes of administration have been published. Oxytetracycline dihydrate (OTC) is a naturally occurring tetracycline, produced by *Streptomyces rimosus*. It was introduced in 1950. It is effective against a wide range of both gram positive and gram negative organisms. The mechanism of action of oxytetracycline is inhibition of protein synthesis. Oxytetracycline dihydrate acts as a chelating agent and chelates calcium, magnesium and aluminium ions in the gut. Oxytetracycline though old, is still widely used for the treatment of bacterial infection in animals due to its promising results. It has broad spectrum of activity, low toxicity and capacity for diffusion into most body fluids and tissues.

Literature is available regarding its pharmacokinetics by intravenous and intramuscular routes in exotic breed of horses, lambs, dogs etc. However, meager information is available on pharmacokinetics of oxytetracycline in indigenous species of cattle, sheep goat and poultry under Indian climatic conditions.

MATERIALS AND METHODS

The study was conducted in six broiler birds, 4-6 weeks age, weighing 1±0.5 kg. All birds were procured from Instructional poultry farm (IPF), Nagla of the University and kept in cage system of animal house of Department of Pharmacology and Toxicology. The birds were kept on pre-experimental period of one month under constant observation before the commencement of experiment to acclimatize them to the new environment. Birds were dewormed with albendazole @ 10mg/kg body weight, one month prior to commencement of experiment. The birds were reared under uniform management and husbandry conditions, maintained on standard ration free of antibiotic and provided water *ad libitum*. All the birds were reared as per the ethical guidelines of CPCSEA with permission from IAEC.

Oxytetracycline, soluble powder (IP) was given as single dose @ 50mg.kg^{-1} oral in poultry birds. The blood samples following single dose (50mg.kg^{-1}) oral administration of oxytetracycline were collected in heparinized tubes through an i.v. cannula placed in the contralateral jugular veins or at wing vein at 0, 5, 15, 30 min. & 1, 2, 4, 8, 12, 24 and 48h after drug administration. Plasma was separated and stored at -30° C till analysis.

Drug extraction from plasma sample was carried out as per the method of Tyczkowska and Aronson, 1986 with slight modification. The collected blood samples were centrifuged at 6000g (10 min) for separation of plasma. 0.5 mL of plasma was pipetted in a microcentrifuge tube and vortex mixed for 30 seconds. 0.5 mL of a 20% acetonitrile, 2%phosphoric acid and 78% deionised water was added to the plasma samples for the precipitation of proteins. The mixture was again vortexed for 30 seconds and was centrifuged at 2,000g for 30 min. The supernatant was then poured into a clean tube and filtered through millipore 0.22 µm filter. An aliquot

of 20 µL of the sample thus obtained was injected into HPLC system for analysis by UV-VIS detector (Shimadzu corporation, Kyoto, Japan).

Separation was achieved using C_{18} reverse phase column, particle size 5 µm (4x 150 mm, Merck) as a stationary phase. An isocratic mobile phase consisted of 20% acetonitrile HPLC grade, 2% phosphoric acid and 78% deionised water HPLC grade. The flow rate was kept at 0.5 mL.min^{-1}. Chromatography was performed at 25^0 C with UV detection at 355 nm. The chromatogram was analyzed by a pharmacokinetic software 'Chromatopak'. Limit of quantification (LOQ) for oxytetracycline was 0.05 µg.mL^{-1}.

RESULTS AND DISCUSSION

A one-compartment model adequately described the plasma concentration-time profile of oxytetracycline in poultry following single dose oral administration. The chromatogram and plasma concentration-time profile following single dose (50 mg.kg^{-1}) oral administration of oxytetracycline in poultry is depicted in Fig 1 and 2. The mean peak plasma concentration of 1.35±0.09 µg.mL^{-1} was attained at 2 h post administration which decreased slowly to a minimum of 0.05±0.04 µg.ml^{-1} at 48h. The

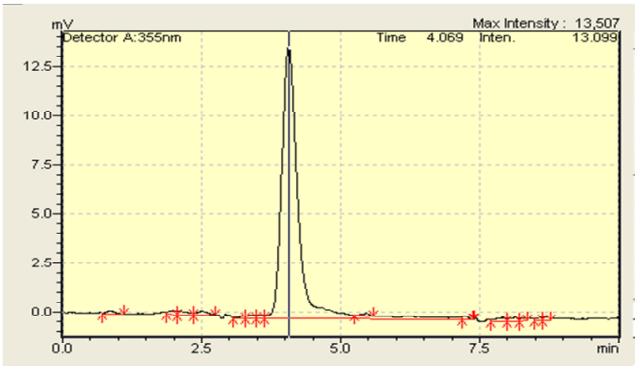

Fig.1:
Chromatogram (HPLC) of oxytetracycline in plasma sample following single dose (50mg.kg^{-1}) oral administration in poultry birds.

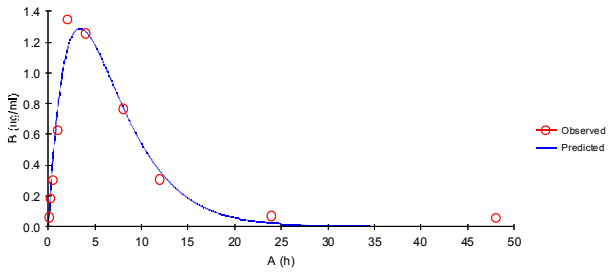

Fig. 2:
Plasma concentration-time plot of observed concentration (mean) Vs predicted profile of Oxytetracycline following single dose (50 mg.kg^{-1}) oral administration in poultry (n=6)

pharmacokinetic parameters describing the disposition kinetics of oxytetracycline following single dose (50 mg.kg^{-1}) oral administration are presented in Table 1.

In the present study, the values of absorption half-life (2.178267 h), elimination rate constant (0.275073 h^{-1}), volume of distribution (16103.43 mL.kg^{-1}), AUC (12.15631 µg.h.mL^{-1}) and time required (2h) to achieve highest concentration suggested the rapid absorption from the site of administration and wide distribution in the body fluids and tissues. The above findings are also in accordance with the findings of Kenneth and Mac Gowan (2006), following 50mg/kg body weight oral administration in broilers with a peak plasma concentration (C_{max}) of 2 µg/ml, time to reach peak conc (T_{max}) 3h, volume of distribution (128 L) and area under curve (AUC) 15.6 µg.h.mL^{-1}.

Elimination half-life (K_{10_HL}) of 2.7379±0.402 h suggested slow elimination from the body. These findings are in accordance with the findings of Craigmill *et al.* (2000), Toutain and Raynaud (1983) and Roncada *et al.* (2000), following i.m. administration in adult and young cattle, however, low value of Vd_{area} and Cl_b were reported by Kumar and Malik (1998), which might be due to variation in the breed, health status of the experimental animals and analytical models.

The therapeutic concentration (0.5 µg.ml^{-1}) with a C_{max} of 1.35 µg.mL^{-1} at 1h was achieved after 2h and maintained for 8h after oral administration in broilers in this study. The AUC value of 12.15631 µg.h.mL^{-1}, suggested the retention of the drug for longer duration which might be due to wide distribution of the drug across the body fluids and tissues after oral administration on account of its lipid solubility. Value of C_{max} in present study following oral route in broilers was found to be 1.35 µg.mL^{-1} at 2h (t_{max}). Based on the pharmacokinetic data, an oral dosage regimen of oxytetracycline with a priming dose of 73.0 mg.kg^{1} (C^{ss}_{min} (minimum steady

Table 1:
Pharmacokinetic parameters of oxytetracycline in plasma following single dose (50mg.kg^{-1}) oral administration in poultry (n=6)

Parameters	Units	Mean±S.E.
K01	1/h	0.34±0.05
K10	1/h	0.27±0.03
K01_HL	h	2.17±0.26
K10_HL	h	2.73±0.40
AUC	h.ug/ml	12.15±0.81
V_F	ml/kg	16103.43±1735.73
CL_F	ml/h/kg	4206.2±278.282
Tmax	h	2.06±0.24
Cmax	ug/ml	1.35±0.09

K01:First order absorption rate constant in one compartment model, K10:First order elimination rate constant in one compartment model, AUC: Total area under the time concentration curve, C_{max}:Peak plasma concentration,T_{max}:Maximum time required to attain peak plasma concentration; Cl_F: Total body clearance, V_F :Volume of distribution.

state concentration) of 0.50 µg.mL^{-1}, C^{ss}_{max} (maximum steady state concentration) of 1.35 µg.ml^{-1}) followed by a maintenance dose of 65.0 mg.kg^{1} at 8 h interval is recommended.

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Research Article

INTRAMUSCULAR PHARMACOKINETICS AND DOSAGE REGIMEN OF CEFEPIME IN BUFFALO CALVES

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ABSTRACT

The disposition kinetics and dosage regimen of cefepime were investigated in buffalo calves following a single intramuscular administration of cefepime (10 mg.kg^{-1} body weight). Pharmacokinetic data after intramuscular administration was best described by one-compartment open model. The peak plasma concentration of cefepime at 45 min was 24.0 ± 0.30 μg.ml^{-1}, which decreased to 0.20 ± 0.003 μg.ml^{-1}at 24 h. The absorption half-life and elimination half-life were 0.17 ± 0.005 h and 2.46 ± 0.004 h, respectively. The apparent volume of distribution (Vd_{area}), total plasma clearance and mean residence time were 0.51 ± 0.003 L.kg^{-1}, 0.139 ± 0.001 L.kg^{-1}.h^{-1} and 3.84 ± 0.01 h, respectively. The systemic bioavailability (F) of cefepime in buffalo calves was 99.6 ± 1.33 per cent. To maintain a minimum therapeutic concentration of cefepime as 1.0 μg.ml^{-1}, a satisfactory dosage regimen of should be 14.8 mg.kg^{-1} repeated at 12 h intrervals.

Key words: Calves, Cefepime, Dosage regimen, Intramuscular, Pharmacokinetics

Introduction

Cefepime is a semi- synthetic, parenteral, fourth generation cephalosporin antibiotic with a broader spectrum of antimicrobial activity than other cephalosporins and non- traditional β-lactam antibiotics. Cefepime shows excellent activity against *Escherichia coli, Klebsiella pneumoneae, Pseudomonas aeroginosa, Enterococcus cloacae, Staphylococcus aureus and Streptococcus* spp. (Baradel and Bryson, 1994). It has variable activity against anaerobic bacteria (Wynd and Paladino, 1996). It is a valuable option in the treatment of lower respiratory tract infections, urinary tract infections, skin infections, bacterial meningitis and provides efficacious antibacterial prophylaxis for biliary tract and prostate surgery with good penetration into cerebrospinal fluid (Tauber *et al.,* 1981). Cefepime pharmacokinetics has also been studied in cow calves (Ismail, 2005; Pawar and Sharma, 2008). Route of administration, dosage regimen and systemic absorption are the important factors, which determine the variation in the intensity and the duration of pharmacological effect. Further the disposition study of cefepime by intramuscular route is essential because this route is frequently employed under field conditions and is easier as well as popular. Keeping this in view, the present study was planned to determine the pharmacokinetics and dosage regimen of cefepime in buffalo calves following intramuscular administration.

MATERIALS AND METHODS

Four healthy male buffalo calves of 6- 12 months age, weighing between 70-100 kg were maintained at Livestock Dairy Farm under uniform conditions for 3 weeks before start of experiment and determined to be healthy by regular clinical examination. The animals were maintained on green fodder, wheat straw and water *ad libitum*. Cefepime hydrochloride was injected intramuscularly at the dose rate of 10 mg.kg^{-1} body weight in 10 % solution with sterilized distilled water. Blood samples (5-6 ml each) were collected from contralateral jugular vein into heparinized test tubes before administration and at 1, 2.5, 5, 7.5, 10, 15, 30, 45, 60 min and 2, 3, 4, 5, 6, 8, 10, 12, 24 and 36 h. Plasma from samples were separated by centrifugation at 3000 rpm for 15 min and stored at –20ºC till analysis. The concentration of cefepime in plasma was estimated by using the microbiological assay technique (Arret *et al.,* 1971) using *E.coli* (MTCC 739) as the test organism. The assay could detect a minimum of 0.1 μg.ml^{-1} of cefepime. The standard curve of cefepime in buffalo calves was linear between 0.25 to 1.5 μg.ml^{-1}. The plasma concentration time data for each calf were determined according to the least squares regression technique. The kinetic parameters were calculated from the formulae derived from one compartment open model (Gibaldi and Perrier, 1982). Based on kinetic data the dosage regimen of cefepime were also determined (Baggot, 1977).

RESULTS AND DISCUSSION

Intramuscular injection resulted into appreciated plasma concentration of cefepime (0.22 ± 0.02 μg.ml^{-1}) at 1 min, which gradually increased and the peak plasma concentration (24.0 ± 0.30 μg.ml^{-1}) was observed at 45 min. The drug was detected in plasma up to 24 h after administration (Fig. 1). In agreement to the present findings, the peak plasma concentration of cefepime

observed in buffalo calves was almost same as observed in goats and calves and lower than ewes and cross-bred calves. The peak plasma concentration of 21.1, 21.7, 31.4 and 33.0 μg.ml^{-1} have been observed in goats (Patani *et al.,* 2006), ewes (Ismail, 2005b) and cross-bred calves (Pawar and Sharma, 2008), respectively following intramuscular administration. A rapid appearance of cefepime in plasma suggests that his drug rapidly enters into systemic circulation following intramuscular administration.

Evaluation of the results on plasma cefepime levels against time indicated that pharmacokinetics of cefepime in buffalo calves after intramuscular administration, was best fitted to one-compartment open model and the pharmacokinetics was described by the following equation:

$$C_p = Be^{-\beta t} - A^{/}e^{-Kat}$$

Where, C_p is the plasma concentration at time t, $A^{/}$ and B are the zero time plasma concentration intercepts of absorption and elimination phases of plasma drug concentration time profile, Ka and β are absorption and elimination rate constants, respectively and e represents the base of natural logarithm. The various pharmacokinetic parameters of cefepime in buffalo calves are calculated and presented in Table 1. The mean values of absorption rate constant and absorption half-life were 3.95 ± 0.10 h^{-1} and 0.17 ± 0.005 h, respectively. The high value of absorption rate constant in the present study, further confirmed that after i.m. administration, the absorption of cefepime was very quick. The rapid absorption following i.m. injection has also been reported in cow calves, buffalo calves, ewes, horses and dogs. The absorption half-life of cefepime in an another study in buffalo calves (Joshi and Sharma, 2007),The calculated value of volume of distribution on the basis of area under plasma concentration-time curve Vd_{area} (0.51 ± 0.003 L.kg^{-1}) in buffalo calves was higher than the reported values in calves 0.23 L.kg^{-1} (Pawar and Sharma, 2008). Despite low Vd_{area} values for cefepime in most species, efficacy against infections located in barrier restricted compartment, such as CNS, has been documented (Grassi and Grassi, 1993). The elimination of cefepime was slow with elimination half-life ($t_{1/2\beta}$) 2.46 ± 0.004 h following i.m. administration in buffalo calves. This low value of elimination half-life supported previous results regarding elimination of cefepime in human, ewes and cross-bred calves. The elimination half-life of cefepime was 2.5 h in human (Hamelin *et al.,* 1993), 2.06 h in ewes (Ismail, 2005) and 1.51 h in cross-bred calves (Pawar and Sharma, 2008). Apparently, the low degree of plasma protein binding and the probable decrease in integrity of blood brain barrier caused by inflammation promote attainment of therapeutic concentration of cefepime in brain tissues. As with other cephalosporins, the extravascular distribution of cefepime was limited to the extracellular fluid (Balant *et al.,* 1985). In spite of its limited distribution into the extravascular fluid as reported in most species studies, it appears that low protein binding and high bactericidal activity are considered the major determinants for promotion of its therapeutic efficacy in all species (Kalman *et al.,* 1992). The values of MRT, Cl_B and AUC after intramuscular administration of cefepime were 3.84 ± 0.01 h, 0.139 ± 0.001 L.kg^{-1}.h^{-1},

Table 2:
Intramuscular dosage regimen of cefepime in healthy buffalo calves at various dosage intervals for microorganisms of different susceptibility.

Microorganisms susceptibility (MIC)[a]	Dosage regimen of cefepime (mg.kg^{-1})				
	Dose	Dosage interval (h)			
		8	10	12	16
0.125	D	0.60	1.05	1.85	5.68
0.25	D	1.20	2.11	3.70	11.3
0.50	D	2.41	4.22	7.39	22.7
1.0	D	4.81	8.44	14.8	45.5
1.5	D	7.22	12.6	22.2	68.2

[a] Values given are expressed as μg.ml^{-1}.

Table 1:
Pharmacokinetic parameters of cefepime in healthy buffalo calves following a single intramuscular dose of 10 mg.kg^{-1} body weight.

Parameter[a]	Unit	Animal number				Mean ± SE
		1	2	3	4	
$A^{/}$	g.ml^{-1}	23.7	24.2	23.5	23.9	23.8 ± 0.13
Ka	h^{-1}	4.20	3.67	3.85	4.10	3.95 ± 0.10
$t_{½Ka}$	h	0.16	0.19	0.18	0.17	0.17 ± 0.005
B	μg.ml^{-1}	21.0	21.9	21.2	21.2	21.3 ± 0.17
β	h^{-1}	0.280	0.278	0.280	0.283	0.280 ± 0.001
$t_{1/2\beta}$	h	2.47	2.47	2.47	2.45	2.46 ± 0.004
AUC	μg.ml^{-1}.h	69.3	71.6	69.6	69.1	69.9 ± 0.50
AUMC	μg.ml^{-1}.h^2	266.5	277.5	268.8	263.3	269.0 ± 2.63
Vd_{area}	L.kg^{-1}	0.51	0.50	0.51	0.51	0.51 ± 0.003
Cl_B	L.kg^{-1}.h^{-1}	0.140	0.139	0.140	0.140	0.139 ± 0.001
MRT	h	3.84	3.87	3.86	3.81	3.84 ± 0.01
F	%	99.7	103.9	97.2	97.6	99.6 ± 1.33

[a] Kinetic parameters are as described by Gibaldi and Perrier (1982).

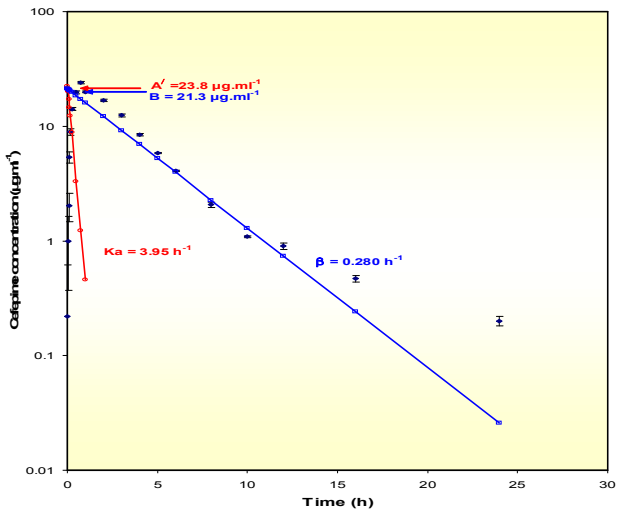

Fig. 1:
Semilogarithmic plot of plasma concentration-time profile of cefepime in healthy buffalo calves following a single intramuscular dose of 10 mg.kg^{-1} body weight.
Data was analyzed according to one-compartment open model. Absorption (Ka) and elimination (β) phases are represented by least square regression lines. The calculated points (o) of the absorption phase were obtained by the residual method. Values denote Mean ± SE of four animals.

69.9 ± 0.50 µg.ml^{-1}.h, respectively.

Among various parameters, bioavailability plays an important role in therapeutic efficacy of a drug. The bioavailability of cefepime in buffalo calves was 99.6 ± 1.33 per cent. The value of systemic bioavailability indicated complete absorption of drug from intramuscular injection site. The systemic bioavailability was reported as 86.8 per cent in ewes (Ismail, 2005), and 95.3 per cent in calves (Joshi and Sharma, 2007). So rapid absorption and higher value of bioavailability revealed that intramuscular administration of cefepime is as good as intravenous injection in treatment of mild to moderate bacterial infections.

The ultimate objective of present study was to determine a satisfactory dosage regimen of cefepime in buffalo calves after its intramuscular administration. It is not axiomatic to compute the dosage regimen of cefepime to be used effectively in clinical practice for the treatment of mild to severe bacterial infections, without having first conducted a detailed pharmacokinetic study. The dosage regimen of cefepime at different minimum therapeutic plasma concentration is given in Table 2. With a minimum therapeutic concentration of cefepime as 1.0 µg.ml^{-1}, which has been shown to be the most effective against majority of sensitive gram positive and gram negative pathogens, the convenient and suitable dosage regimen of cefepime after intramuscular administration would be 14.8 mg.kg^{-1} repeated at 12 h intrervals.

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Research Article

SPINOSAD: SUB-ACUTE IMMUNOTOXICITY STUDIES IN MICE

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ABSTRACT

In the present study, the immunological alterations induced by spinosad were assessed by measuring effects on sub-acute exposure of spinosad on humoral immune response and cell mediated immune response in Swiss albino male mice. The serum antibody titer against sheep RBCs (SRBC) was measured by haemagglutination (HA) test to assess the effect of spinosad treatment on humoral immune response.The results of HA test indicated significant effect on antibody titer against SRBC. There was progressive and proportionate decrease in antibody titer, significant decrease at both dose levels of spinosad treatment.The effect on cellular immune responsewas assessed with the help of two important parameters, Phytohemagglutinin (PHA) induced lymphocyte proliferation assay and delayed type hypersensitivity (DTH) response to SRBC.Stimulation index (S.I.) of lymphocyte proliferation assay did not show any biologically significant difference in spinosad treated groups as compared to saline treated group.DTHresponse to SRBC was characterized by mild to moderate inflammatory reaction, with erythema, edema, swelling in saline treated mice group.The findings of DTH response clearly indicate that spinosad has mild immunotoxic effect at higher dose i.e. 58 mg/kg/day; i.p. and has mildly suppressed DTH response at this dose level.

Key words : Spibosad, immunotoxicity, mice.

INTRODUCTION

Bio-pesticides are typically produced by growing and concentrating naturally occurring organisms and/or their metabolites including bacteria and other microbes, fungi, nematodes, proteins etc.Spinosad is a new class of insecticides, used to control a variety of insect pests, including fruit flies, caterpillars, leaf miners and thrips, sawflies, and leaf beetles. Spinosad is recommended for use in an Integrated Pest Management (IPM) program and it does not significantly affect beneficial organisms including ladybugs, green lacewings, minute pirate, bugs and predatory mites. Its favorable mammalian and environmental profile, insect selectivity and IPM fit, unique mode of action and resistance management properties and outstanding efficacy are resulting in rapid registration and adoption by growers around the world documenting increased agricultural produce.

Till date, only preliminary toxicological information is available on spinosad and investigations in the literature show very few pharmacological and toxicological studies on spinosad with no reports available on the immunotoxicity of spinosad. Hence, the proposed investigation was undertaken with an objective to evaluate the immunotoxic potential of spinosad. The information obtained from this investigation will help in understanding the adverse effects of spinosad, if any, on exposure of animals and human beings and thereby will help in prevention and treatment of poisoning and assessing safety for its widespread use.

MATERIALS AND METHODS

The study was conducted on male Swiss albino mice. The formulation product of spinosad, Tracer (Spinosad 45% SC), Dow AgroSciences India Pvt. Ltd., procured from the local market in Hisar was used in this study.

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*. The animals were acclimatized to laboratory conditions for 3-4 days before the experiments were conducted. The prior approval of Institutional Animal Ethical Committee for the protocol of this study was obtained.

Chemicals and Reagents

Iscove's Modified Dulbecco Medium (IMDM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen. Dexamethasone, Cyclophosphamide, Phytohemagglutinin (PHA), Phenazinemethosulphate (PMS), XTT dye, Dulbecco's Phosphate Buffer Saline (DPBS), Antibiotic and Antimycoticsolution, Freund's Complete Adjuvant (FCA) were purchased from Sigma Aldrich (India). Plastic wares such as centrifuge tubes (15 ml and 50 ml), microcentrifuge tubes (0.7 ml) and micro tips (2-200 µl and 200-1000 µl) were procured from Genetix. F96 MAXISORP Nunc-Immuno Plates were purchased from Nunc.

Dose selection

The doses selected for administration were

derived from the maxmium tolerated dose of spinosad. The immunological 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, intraperitoneally and thus the selected doses were 29 mg/kg and 58 mg/kg; i.p. of spinosad, respectively).

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 two control groups, one negative control group, in which normal saline at a dose rate of 10 ml/kg body weight was administered daily to a group of 6 male mice i.p. for a period of 28 days and one positive control group which received cyclophosphamide @ 55 mg/kg body weight, i.p. in HA titer and lymphocyte proliferation assey (LPA) experiment for 5 days (from day 24th of drug administration, unless mentioned otherwise) and dexamethasone @ 10mg/kg body weight, i.p. in DTH response experiment for 5 days (from day 17th of drug administration). The test and control groups of animals were sacrificed on day 29 of the experiment.

Effect of Humoral Immune Response (Haemagglutination (HA) titer (Collection of sheep blood and separation of sheep red blood cells)

Sheep blood was collected in sterile centrifuge tube containing sterile Alsever's solution with the help of sterile syringe and needle (18 G) from jugular vein of sheep. Sheep blood and Alsever's solution were taken in the ratio of 1: 2.Alsever's solution was prepared by mixing Glucose (2.050 g); Sodium chloride (0.420 g); Sodium citrate (0.800 g) and Citric acid (0.055 g) in distilled water. Distilled water was added to make volume 100 ml and autoclaved at 10 lb pressure for 15 min and then stored at 4^{0} C. Sheep blood (mixed in Alsever's solution, about 4 ml) was washed thrice in Dulbecco's Phosphate Buffer Saline (DPBS), at 1500 rpm X 10 min. From final RBC pack, 1:10 and 1:100 dilutions were prepared for the purpose of counting RBCs. Final volume of RBCs was adjusted as $1.5x10^{9}$ cells/ml.

Mice immunization

For evaluation of HA titer, mice from this experimental set up were immunized by i.p. injection of SRBCs ($4.5x10^{8}$ cells/mice, 0.3 ml/mice) in saline, ten days before completion of the treatment period (Elsabbagh and El-tawil, 2001).

Titration of haemagglutinating serum antibodies

At the end of experiment, sera samples were prepared from peripheral blood samples of each group of immunized mice and decomplemented by heating at 56^{0}C for 30 minutes. These sera samples were then kept at –20^{0}C until used for estimation of antibody titer. To prevent non-specific agglutination, 1% Bovine Serum Albumin (BSA) was added in DPBS and then this DPBS was used for preparing 1% (v/v) SRBC suspension.

The microtitre HA technique as described by Puri *et al.* (1994) was employed to determine the serum antibody titer. A volume of 50 µl of 0.15 M phosphate buffer saline (PBS, pH 7.2) was placed into all the 96 wells of round bottom microtitre plate (Laxbro Manufacturing Company, Pune, India). Then 50 µl of test serum was added to the first well, mixed thoroughly and transferred serially up to the 12th well by double dilution technique and 50 µl of content was discarded from 12th well. Then 50 µl of 1% (v/v) sheep RBC suspension was added in all the wells. Last row served as SRBC control which contained only PBS and SRBC but no test serum. Plate was then mixed by tapping and incubated at 37^{0} C for 1.5 – 2 hours and then observed for haemagglutination pattern. The reciprocal of the highest dilution giving complete haemagglutination was taken as the antibody titer. Results were represented as the $\log_2$ value of the reciprocal of serum dilution giving haemagglutination.

Delayed type hypersensitivity (DTH) response

The DTH response was assessed by the method of Gokhale*et al.* (2003) using SRBC as antigen with some modifications. For this experimental set up, dexamethasone was used for the positive control group animals and administered for 5 days (Hassan *et al.,* 2004).

Sensitization of mice

On 19th day of the treatment (in negative control group and spinosad treated groups and 3rd day of dexamethasone administration in positive control group), mice were sensitized by s/c injection of SRBC suspended in Freund's complete adjuvant (FCA) on their back (in neck region) with the help of 1 ml plastic tuberculin syringe and 26 G needle (50µL SRBC suspension in FCA). SRBCs were separated from blood as mentioned above. To prepare SRBC-FCA emulsion, equal volume of SRBC and FCA (2.5 ml of each, sufficient to be injected for 40 mice) was taken. In a vial containing 2.5 ml of FCA, small quantity of SRBC suspension (100 to 500 µl) of the total 2.5 ml was added and with the help of 16/18 G needle and 5 ml syringe it was mixed vigorously. Resultant emulsion was tested by putting one drop in beaker containing cold water and watched for its spreading, ideally this drop should not spread on water surface, if it spreads, then emulsion should be again mixed vigorously until its consistency becomes thick.

Challenge

After 10 days (i.e. 29th day of treatment), sensitized mice were challenged by injecting 50 µl of SRBC in right hind foot pad with the help of 26 G needle and tuberculin syringe. Before injecting SRBC in foot pad, mice were lightly anaesthetized by i.p. injection of ketamine HCl (50 mg/kg body weight).

Assessment of reaction

Swelling in the right hind foot pad was measured by pressure sensitive micrometer screw gauge (Mitutoyo, Japan), 24 and 48 hours after the challenge. Histopathology of footpad was also performed to study cellular changes.

Lymphocyte proliferation assay (LPA)

Lymphocyte proliferation assay was performed by the method of Roehm*et al.* (1991) and Neishabouri*et al.*(2004) with some modifications. Cyclophosphamide was administered to the mice of positive control group for 5 days concurrent with day 24 of spinosad treatment groups.

Preparation of cell culture medium

Iscove's Modified Dulbecco Medium (IMDM) was used as cell culture medium. It was distributed in aliquots of 50 ml in sterile centrifuge tubes and kept at 4^0C until use. All the handling/procedures were performed in laminar flow. Media was supplemented with sterile 10% fetal bovine serum (FBS), 1% Antibiotic&Antimycotic stock solution. Washing of lymphocytes was done with medium containing only antibiotics but no FBS; however, after final washing lymphocytes were suspended in medium containing FBS i.e. complete IMDM.

Isolation of splenic lymphocytes

On 29th day, mice were euthanized with the help of chloroform, placed on right abdomen on tissue paper in laminar flow. Body coat of mouse was thoroughly wetted with spirit and with the help of syringe and forceps, small incision was given on right abdomen, spleen was removed aseptically and put in a Petri dish containing cool medium. With the help of sterile syringe hub and nylon mesh, spleen was processed and made into spleen single cell suspension by removing the connective tissue and capsule. This splenocytes suspension was transferred and centrifuged for 5 minutes, 4^0 C at 1000 rpm. The supernatant was discarded and pellet was suspended with RBC lysis buffer to omit RBCs.After storage for 5-7 minutes in cold water, the suspension cells were washed twice with IMDM and centrifuged for 5 minutes, 4^0C at 1000 rpm. The final cell pellet thus obtained was resuspended in the complete medium for the assay being performed.

Standardization

Concentration of PHA and concentration of splenic lymphocytes to be used in the assay was standardized. For this purpose, mice which had not been subjected to any chemical/drug treatment were used. The cells pellet obtained after last washing were resuspended in complete medium and were used as neat and 1:2 dilution. PHA was dissolved in IMDM so as to make 1 mg/ml stock under sterile conditions and aliquots of 1 ml were stored at -50^0 C until used. Each dilution of the samples was cultured in triplicate with or without six different concentrations of PHA in a flat bottom 96 well tissue culture plates. Cell suspension was added @ 200 µl/well and six different concentrations of PHA i.e. 5, 10, 20 25, 50 and 100 µg/ml were made from 1 mg/ml stock. These six concentrations of PHA were added as 20 µl/well in a 200 µl of cell suspension. To the mitogen negative wells (cells only control), 20 µl of the medium was added. The plate was covered with lid and transferred in humidified CO_2 incubator at 37^0 C, in 5% CO_2 for 72 hours. After 3 days of incubation, culture wells were pulsed with fresh XTT dye solutions combined with phenazinemethosulphate (PMS), an electron coupling reagent which enhances bioreduction of XTT dye by murine cells/cell line.

XTT solutions were made fresh every day by dissolving in warm media at 1 mg/ml at 40^0C in a water bath. This tetrazolium salt was filtered (0.2 µm, Millipore® filter) immediately prior to use. PMS was made up as 10 mM solution in IMDM (3.063 mg/ml of IMDM) and stored at 4^0C for 3-5 days. PMS was added to the XTT solution (2.5 µl of PMS/ml of XTT solution) immediately before use (Goodwin *et al.,* 1995). Fifty µl of XTT/PMS were added per well of 200 µl culture. Plates were gently shaken to mix the contents and incubated for 3-4 hours at 37^0C. The color developed in the wells was determined using an UVmax kinetic microplate reader (Tecan) at absorbance of the 450 nm with reference wavelength of 650 nm. Before taking reading, plates were shaken for 10 seconds on plate shaker (REMI Instruments Ltd., Mumbai, India). Out of the six concentrations of PHA, 25µg/ml was found to be optimum and gave A_{450} values significantly different from the control wells containing only cells. Also, at this concentration of PHA, A_{450} value of wells containing '1:2 diluted cells' was coherent and reproducible than those of the wells containing 'neat cells'. So, $2x10^6$ cells/ml were found to be optimum when used in 200 µl/well (i.e. $4x10^5$ cells/well).

Lymphocyte proliferation response in splenocytes cultures

Single spleen cell suspensions without red blood cells were prepared as noted above. After last wash, the cells were resuspended in IMDM containing sterile 10% fetal bovine serum (FBS), 1% Antibiotics and antimycotic solution, i.e. complete IMDM. Cell viability was determined by 0.4 % trypan blue dye exclusion test. Finally the viable cells number was adjusted to $2x10^6$ cells/ml with the help of complete medium. The test was performed in flat bottom 96 wells tissue culture plates. Triplicate cultures of each sample were made with and without mitogen. Lymphocyte cell suspension was added @ 200 µl/well and then 20 µl of 25 µg/ml of PHA representing 0.5 µg/well or culture was added. To the wells containing no mitogen (cells only

control), 20 µl of medium was added. Corner rows and columns were filled with 220 µl of medium which apart from serving medium control, helped in tackling evaporation of well contents. The plate was covered with lid and transferred to humidified CO_2 incubator at 37^{0}C, 5% CO_2 for 72 hours. After 3 days of incubation, the number of proliferating cells was determined with XTT dye combined with phenazinemethosulphate(PMS).

Fifty µl of XTT/PMS were added to each 200 µl of culture. Plates were gently shaken to mix the contents. After additional 3-4 hours of incubation at 37^{0}C, the absorbance of the wells was determined using UVmax kinetic microplate reader at a test wavelength of 450 nm with reference wavelength of 650 nm. Before taking reading, plates were shaken for 10 seconds on plate reader. Stimulation index (S.I.) was calculated as absorbance of the sample stimulated by PHA divided by absorbance of the sample without stimulation by PHA.

Spleen cell cellularity

Spleen cell cellularity was determined at the time of lymphocyte proliferation assay by counting with a hemocytometer after preparing spleen single cell suspension followed by red blood cell lysis.

2.4 Histopathological studies

At the end of experiment mice were sacrificed, macroscopic lesions infootpad were recorded and representative pieces of tissue from these 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) stain (Luna, 1968).

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

The results of investigation on immunotoxic effects of Spinosad in mice are as follows:

Effect on humoral immune response / Haemagglutination (HA) titer

The effect of spinosad on haemagglutination (HA) titer in Swiss albino male mice is presented in Table 1 and depicted graphically in Fig 1. Haemagglutination titer against SRBC, a T cell dependent antigen is expressed as $\log_2$ antibody titer. The spinosad treatment caused a dose dependent and significant decrease in serum antibody titer, with higher dose causing highly significant decrease in serum antibody titer as compared to saline treated group. In cyclophosphamide treated group also serum antibody titer decreased as compared to saline treated group, the difference being highly significant. The $\log_2$ antibody titer levels were 7.60 ± 0.24, 5.33 ± 0.42, 4.33 ± 0.42 and 3.33 ± 0.56 for saline group, lower dose of spinosad, higher dose of spinosad and for cyclophosphamide, respectively.

Effect on cell mediated immune response

Delayed type hypersensitivity (DTH) response

The effect of spinosad on delayed type hypersensitivity (DTH) in Swiss albino male mice is presented in Table 2 and depicted graphically in Fig 2. In evaluation of DTH response to SRBC, maximum increase in paw thickness (in mm) was measured after 24 hours and 48 hours of challenge and calculated as percent increase in paw thickness and expressed as % DTH response. Delayed type hypersensitivity (DTH) response to SRBC was characterized by mild to moderate inflammatory reaction, with erythema, edema, swelling in saline treated mice group. There was increase in paw thickness after injecting challenging dose of SRBCs in all groups, maximum thickness being after 24 hours.

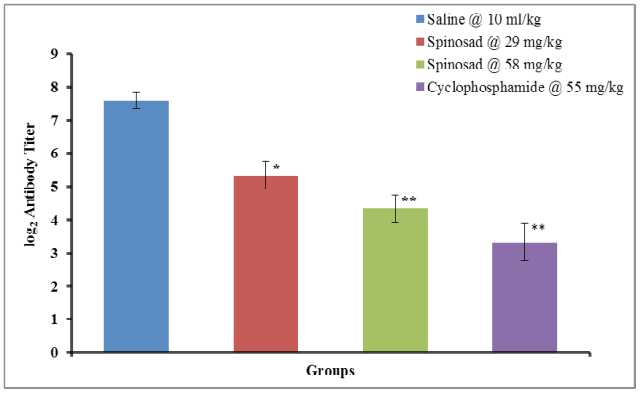
The spinosad treatment caused a dose-dependent decrease in the gross symptoms and inflammatory reaction. Mice in spinosad treated higher

Table 1:

Effect of spinosad on haemagglutination (HA) titer in Swiss albino male mice

Treatment	$\log_2$ Antibody Titer
Saline @ 10 ml/kg	7.60 ± 0.24
Spinosad @ 29 mg/kg	5.33 ± 0.42*
Spinosad @ 58 mg/kg	4.33 ± 0.42**
Cyclophosphamide @ 55 mg/kg	3.33 ± 0.56**

Data presented as Mean ± SE; (n =6 mice per group)
* Values differ significantly from control (P≤0.05)
** Values differ significantly from control (P≤0.01)



Vertical bars over columns indicate standard error of mean
* Values differ significantly from control (P≤0.05)
** Values differ significantly from control (P≤0.01)

Fig 1:

Effect of spinosad on haemagglutination (HA) titer in Swiss albino male mice

dose group and dexamethasone treated group showed very mild inflammatory reaction with very mild edema and swelling and the DTH response in these groups was significantly lower as compared to saline treated group at 24 hours and 48 hours after challenge.

Lymphocyte proliferation assay (LPA)

The effect of spinosad on lymphocyte proliferation assay (LPA) in Swiss albino male mice is presented in Table 3 and depicted graphically in Fig 3. The results of the lymphocyte proliferation test are expressed as phytohaemagglutinin (PHA) induced stimulation index. Spinosad treatment decreased the lymphocyte proliferation in response to PHA as compared to saline treated group, though the effect was non-significant. In cyclophosphamide treated group stimulation index was significantly decreased as compared to saline treated group.

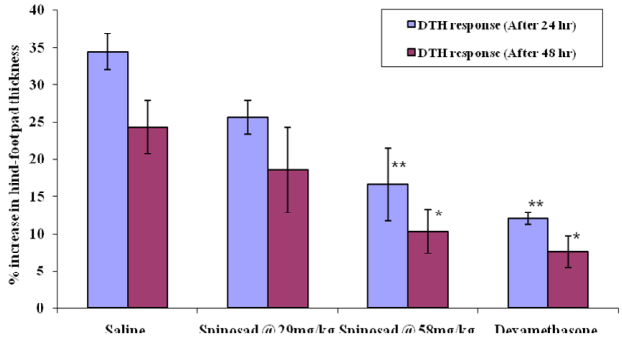
Spleen Cell cellularity

The effect of spinosad on splenic cellularity in Swiss albino male mice is presented in Table 4 and depicted graphically in Fig 4. Splenocytes in splenic single cell suspension from the mice of each group were counted and mean spleen cell count was expressed as $x10^6$/ml cells. Spinosad treatment decreased splenic cellularity in both treatment groups in dose dependent manner, though it was non-significant. Cyclophosphamide treatment caused highly significant decrease in splenic cellularity.

Table 2:
Effect of spinosad on Delayed type hypersensitivity (DTH) response in Swiss albino male mice

Treatment	DTH response (% increase in hind-foot pad thickness)	
	After 24 hr	After 48 hr
Saline @ 10 ml/kg	34.47 ± 2.39	24.32 ± 3.62
Spinosad @ 29 mg/kg	25.64 ± 2.29	18.57 ± 5.75
Spinosad @ 58 mg/kg	16.61 ± 4.85**	10.36 ± 2.99*
Dexamethasone @ 10 mg/kg	12.08 ± 0.90**	7.65 ± 2.08*

Data presented as Mean ± SE; (n = 6 mice per group)
* Values differ significantly from control (P≤0.05)
** Values differ significantly from control (P≤0.01)



Vertical bars over columns indicate standard error of mean
* Values differ significantly from control (P≤0.05)
** Values differ significantly from control (P≤0.01)

Fig 2:
Effect of spinosad on Delayed type hypersensitivity (DTH) response in Swiss albino male mice

Table 3:
Effect of spinosad on lympho-proliferative response to PHA in Swiss albino male mice

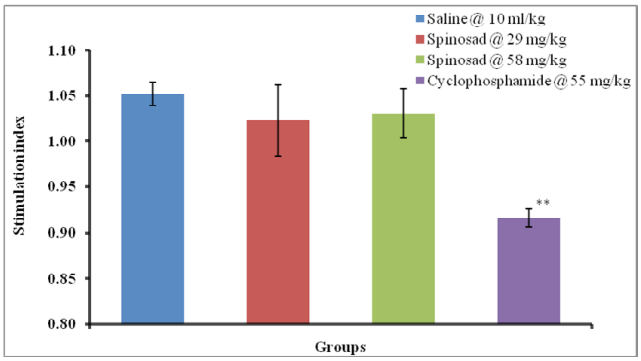
Treatment	Stimulation Index	n
Saline @ 10 ml/kg	1.052 ± 0.013	3
Spinosad @ 29 mg/kg	1.023 ± 0.039	5
Spinosad @ 58 mg/kg	1.031 ± 0.027	5
Cyclophosphamide @ 55 mg/kg	0.916 ± 0.01**	5

Data presented as Mean ± SE; (n = mice per group)
** Values differ significantly from control (P≤0.01)

Table 4:
Effect of spinosad on splenic cellularity in Swiss albino male mice

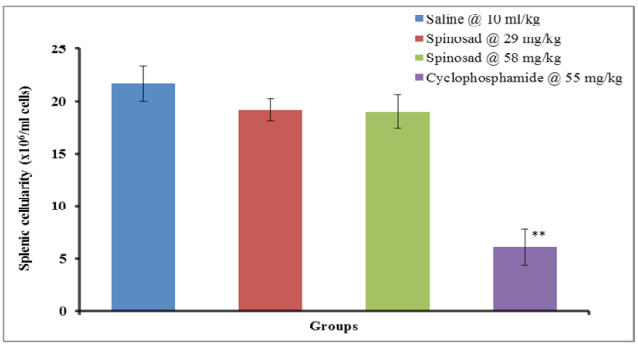
Treatment	Splenic cellularity ($X10^6$/ml cells)	N
Saline @ 10 ml/kg	21.67 ± 1.67	3
Spinosad @ 29 mg/kg	19.20 ± 1.02	5
Spinosad @ 58 mg/kg	19.00 ± 1.64	5
Cyclophosphamide @ 55 mg/kg	6.08 ± 1.75**	5

Data presented as Mean ± SE; (n = mice per group)
** Values differ significantly from control (P≤0.01)



Vertical bars over columns indicate standard error of mean
** Values differ significantly from control (P≤0.01)

Fig 3:
Effect of spinosad on Lympho-proliferative response to PHA in Swiss albino male mice



Vertical bars over columns indicate standard error of mean
** Values differ significantly from control (P≤0.01)

Fig 4:
Effect of spinosad on splenic cellularity in Swiss albino male mice

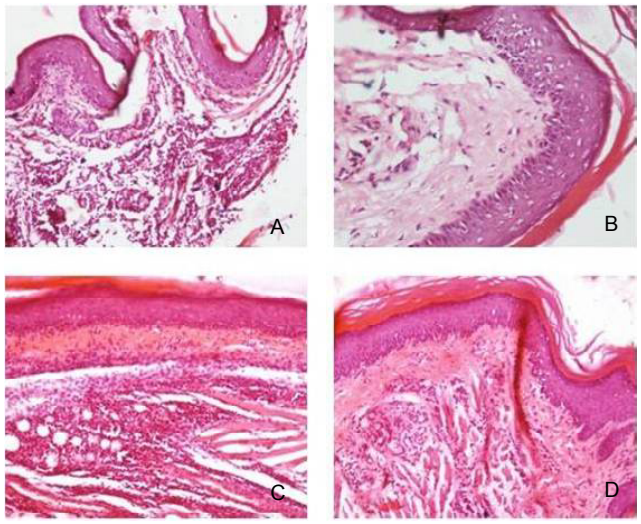


Fig. 5:
Microphotograph of foot pad section of Swiss albino male mice (x 400).

A: Foot pad section of mice of vehicle control group showing severe mononuclear cells infiltration in dermis

B: Dexamethasone treated positive control group (10 mg/kg; i.p. x 5 days) showing very mild inflammatory reaction suggesting immunosuppression.

C: Spinosad treated higher dose group (58 mg/kg; i.p.) showing mild inflammatory reaction with mononuclear cells infiltration in dermis portion of foot pad.

D: Spinosad treated lower dose group (29 mg/kg; i.p.) showing very mild inflammatory reaction with few mononuclear cells infiltration in dermis portion of foot pad.

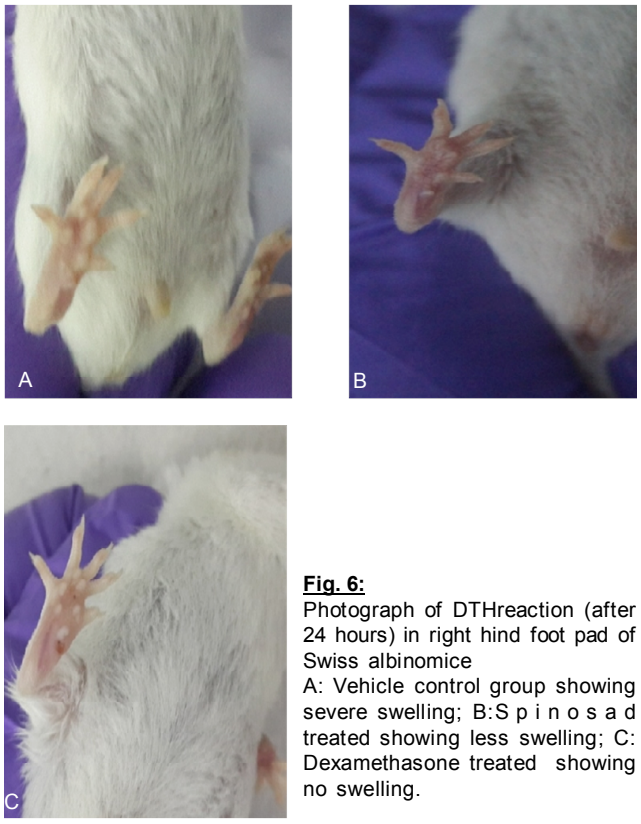


Fig. 6:
Photograph of DTHreaction (after 24 hours) in right hind foot pad of Swiss albinomice
A: Vehicle control group showing severe swelling; B:Spinosad treated showing less swelling; C: Dexamethasone treated showing no swelling.

Histopathological studies

Histopathological examination of foot pad was done for evaluating cellular changes in DTH response and revealed mild pathological alterations with infiltration of mononuclear cells in dermis in spinosad treated groups (Fig 5). Foot pad sections of mice from dexamethasone treated dose group revealed very mild inflammatory reaction suggesting immunosuppression (Fig 6).

DISCUSSION

The immunotoxicity of spinosad insecticide were studied in Swiss albino male mice in this investigation. For the investigation of potential effects of compounds on the immune system, a tiered approach is recommended. In tier 1, general toxicity studies are conducted with emphasis on evaluation of organs belonging to the immune system and it includes organ weights and histopathological evaluation of immune organs like spleen, thymus, lymph nodes, blood and bone marrow which may detect the occurrence of direct immunotoxicity. Tier 2 studies are done to investigate the effects of xenobiotics on immune functionality in immunological challenge experiments. Tier 2 studies can determine the extent of the immunosuppression and identify which specific part(s) or cellular component(s) of the immune system is involved.

For rats, these studies have been described in OECD (Organisation for Economic Co-operation and Development, Paris, France) guideline (TG 407). Accordingly, in the present investigation, a total of four groups were formed including one vehicle control group (mice receiving normal saline), two test groups comprising higher and lower toxic dose of spinosad and one positive control group (mice receiving cyclophosphamide or dexamethasone). The effect of administration of spinosad daily for 28 days at the dose rate of 58 mg/kg (1/10th of MTD) and 29 mg/kg (1/20th of MTD) on humoral immune response, cell mediated immune response, and histopathology of foot pad in Swiss albino male mice was investigated in the present study.

Immunotoxicology can be most simply defined as the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, and, in some instances, biological materials. Studies in animals and humans have indicated that the immune system comprises potential target organs, and that damage to this system can be associated with morbidity and even mortality. Indeed, in some instances, the immune system has been shown to be compromised (decreased lymphoid cellularity, alterations in lymphocyte sub-populations, decreased host resistance, and altered specific immune function responses) in the absence of observed toxicity in other organ systems.

If the primary defenses against infection (innate

to an antigen, which may act to opsonize or neutralize the invader, or the antibodies act to recruit other factors, such as the complement cascade. CMI is that part of the immune system in which various effector cells perform a wide variety of functions to eliminate invaders. Often, these two branches are coordinated, such as activation of CMI in the form of a T-helper cell produces specific cytokines that enhance B-cell proliferation and differentiation to produce more antibodies.

In the present study, the serum antibody titer against sheep RBCs (SRBC) was measured by haemagglutination (HA) test to assess the effect of spinosad treatment on humoral immune response. SRBC is a T cell dependent antigen. The results of HA test indicated significant effect on antibody titer against SRBC. There was progressive and proportionate decrease in antibody titer, significant decrease at both dose levels i.e. at lower dose level and higher dose level of spinosad treatment. Similarly, cyclophosphamide treated mice group exhibited severely decreased antibody titer which was even significant at 1% level of significance. Kawani (2007) and Badgujar (2010) have reported dose dependent decrease in antibody titer of imidacloprid treated dose groups while evaluating immunotoxic effect of imidacloprid in BALB/c mice. Gatne*et al.* (2006) also reported progressive and proportional decrease in haemagglutination antibody titer in Sprague-Dawley rats treated with different doses of technical grade imidacloprid. Banerjee *et al.* (1996) reported significant influence of sub-chronic exposure of lindane on humoral immunity in mice. Lindane suppressed both primary and secondary antibody responses to SRBC, the effects being more pronounced on the secondary than primary response. Willtrout*et al.* (1978) found significant suppression of the humoral immune response following orally administered pesticides (Carbaryl, DDT, Parathion, Chlordneform and Ametryne) in mice at near lethal doses. The primary and secondary hemolysin titers against SRBCs were reduced to 62% and 63% of control values for cupravit and 76% and 76% of control values for pervicur (Elsabbagh and El-tawil, 2001).

The effect of subacute exposure of spinosad on cellular immune response in Swiss albino male mice was assessed with the help of two important parameters, PHA induced lymphocyte proliferation assay and delayed type hypersensitivity (DTH) response to SRBC. Lymphocyte proliferation assay was performed using PHA as a mitogen and XTT dye as tetrazolium salt (in PMS) was used for a colorimetric assay in evaluation of proliferation of T cell. Many workers have used MTS/PMS and MTT dye instead of PMS and XTT. MTS/PMS is comparatively very new dye and is very much costly and MTT gives insoluble blue colouredformazan product, which needs to be dissolved before spectrophotometric evaluation.

Stimulation index (S.I.) of lymphocyte proliferation test did not show any biologically significant difference in spinosad treated groups as compared to saline treated group. However, cyclophosphamide treated group showed significantly decreased S.I. compared to saline treated group. Badgujar (2010) reported significantly decreased S.I. in highest toxic dose group of imidacloprid and cyclophosphamide treated mice group. Decrease in lymphocyte stimulation is an indication of suppression of T helper cell population which is involved in cell mediated immune responses. Diazinon only at highest toxic dose i.e. 25 mg/kg suppressed lymphocyte proliferative response to 5 µg/ml of PHA, while other two toxic dose groups did not show any change in stimulation index (Neishabouri *et al.,* 2004). No significant changes in spleen lymphocytes proliferative response to phytohaemagglutinin (PHA, 5µg/ml) were observed in C57BL/6 mice treated with different doses of propoxur (Hassan *et al.,* 2004). Pirimiphos-methyl significantly decreased proliferative activity of spleen lymphocytes in BALB/c mice at all the 3 doses checked (Kim *et al.,* 2007). Peden-Adams *et al.* (2007) also evaluated splenic lymphocyte proliferation using the tritiated thymidine method in adult female B6C3F1 mice after 14 days and 28 days oral exposure to sulfluramid (a per-fluorinated hydrocarbon). They found that exposure to sulfluramid for 14 or 28 days did not significantly alter either T- or B-cell mitogen induced proliferation, suggesting that lymphocytic proliferative responses were normal.

Delayed type hypersensitivity (DTH) response to SRBC was characterized by mild to moderate inflammatory reaction, with erythema, edema, swelling in saline treated mice group. There was increase in paw thickness after injecting challenging dose of SRBCs in all groups, maximum thickness being after 24 hours. The spinosad treatment caused a dose dependent decrease in the gross symptoms and inflammatory reaction. Mice in spinosad treated higher dose group and dexamethasone treated group showed very mild inflammatory reaction with very mild edema and swelling and the DTH response in these groups was significantly lower as compared to saline treated group at 24 hours and 48 hours after challenge.

The increase in paw thickness (in mm) was measured 24 hours and 48 hours after challenge and calculated as percent increase in paw thickness which is then expressed as % DTH response. Percent DTH response decreased non-significantly in lower dose group after 24 hours and 48 hours, but in higher dose group and dexamethasone treated mice group, it was suppressed significantly as compared to control.

Histopathological examination of foot pad was done for evaluating cellular changes in DTH response and revealed very mild pathological alterations with infiltration of leukocytes/mononuclear cells in dermis in spinosad treated groups. Foot pad sections of mice from dexamethasone treated group revealed very mild inflammatory reaction suggesting suppression of DTH response. Foot pad sections of saline treated mice group showed mild reaction and relatively large number of inflammatory cells such as macrophages, lymphocytes along with few neutrophils/polymorphonuclear cells were present in dermis. Intensity of reaction in dexamethasone treated mice group was still lower/milder than that of highest toxic group suggesting marked suppression of DTH response.

The findings of DTH response clearly indicate that spinosad has mild immunotoxic effect at higher dose i.e. 58 mg/kg/day; i.p. and has mildly suppressed DTH response at this dose level. DTH response involves T effector cells (Danneberg, 1991) and its suppression at higher dose of spinosad suggests that spinosad has direct cytotoxic effect on T effector cells at higher dose level. Following interaction with a specific antigen, the effector cells are responsible for elaboration of lymphokines. In the DTH reaction, the primary lymphokine response involved appears to be responsible for the accumulation of mononuclear cell infiltrates, mononuclear cell interaction and increased vascular permeability that occur in the vicinity of stimulus. Neishabouri*et al.* (2004) also reported suppression of DTH reaction evaluated against SRBC at highest and middle toxic dose (25 mg/kg/day and 2 mg/kg/day for 28 days) of diazinon in C57BL/6 mice. High doses of propoxur (10 mg/kg) also suppressed the DTH response 24 hours after secondary injection of antigen (SRBCs); however there was no significant change in DTH response after 48 hours of injection at this dose rate. Propoxur at lower doses (0.2 and 2 mg/kg) did not change DTH response after 24 and 48 hours of challenge (Hassan *et al.,* 2004). Cupravit and pervicur fungicides evaluated for immunotoxic potential have also significantly inhibited DTH reaction to tuberculin revealing suppression of cell-mediated immune response (Elsabbagh and El-tawil, 2001). Gatne*et al.* (2006) also reported that imidacloprid at highest toxic dose (160 mg/kg/day) decreased DTH response against SRBC in Sprague-Dawley rats which was not significant statistically.

There are two basic mechanisms by which xenobiotics may induce suppression of the immune system: (1) by direct action of the xenobiotics upon the lymphoid organs or cells involved in the immune response and (2) by indirect action of the xenobiotics on other organ or physiological systems, such as neuroendocrine interactions, metabolic activation of xenobiotics to toxic metabolites or hepatic modulation which then impact immune response (Ladics and Woolhiser, 2007). Significant reduction in spleen cell cellularity in cyclophosphamide treated mice group was quite obvious since, it is a known immunosuppressive agent, which causes general reduction in lymphocytes as well as alterations in lymphocyte function. Mechanism of action of cyclophosphamide is indirect, that involves metabolic activation or bio-activation. Cyclophosphamide is a prototypical member of a class of drugs known as alkylating agents. Upon entering the cell, the inactive, non-cytotoxic drug is converted into phosphoramide mustard, a DNA alkylating agent that inhibits cell replication (Shand, 1979). Mild decrease in spleen cell cellularity along with mild decrease in relative weight of spleen in spinosad treated higher and lower dose group animals suggests mild splenic cell damage or cell death which might be attributed to spinosad or its metabolites. Since spleen is important for immune functions, histological changes in spleen may reflect as dysfunction. Histopathological evaluation of spleen revealed dose dependent mild depletion of lymphocytes in white pulp of spleen and confirms our findings of mildly decreased spleen cell cellularity and reduced relative weight of spleen. Kim *et al.* (2007) have reported that sub-chronic oral exposure with pirimiphos-methyl at 120 mg/kg/day for 28 days produced significant decrease in relative spleen weight and splenic cellularity in BALB/c mice. Gatne*et al.* (2006) evaluated immunotoxic effects of subacute exposure of imidacloprid in Sprague-Dawley albino rats and reported decrease in cellular immunity which was evident from decrease in phagocytic index, decrease in lymphocyte migration inhibition test and decrease in DTH response.

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Research Article

CURCUMIN ENHANCES THE EXPRESSION OF GROWTH ASSOCIATED PROTEIN-43 (GAP-43) IN HEALING TISSUE OF DIABETIC RATS

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ABSTRACT

In our earlier studies, we have reported that that topical application of curcumin caused faster healing of cutaneous wounds in diabetic rats which was might be due to their antioxidant, anti-inflammatory and angiogenic potentials. The aim of this study was to examine the effect of topical application of curcumin on neuronal growth in healing tissue in streptozotocin-induced diabetic rats. Excision cutaneous wound was created in diabetic rats and wounded rats were divided into three groups *viz.* i) control, ii) pluronic F-127 gel-treated and iii) curcumin-treated. The pluronic F-127 gel (25%) and curcumin (0.3%) in pluronic gel were topically applied once daily for 19 days. Five rats were killed on days 3, 7, 14 and 19 from each group and granulation/healing tissue was collected and processed for immunohistochemical analysis of Growth Associated Protein-43 (GAP-43) positive nerve fibers. The curcumin treatment increased GAP-43 positive nerve fibers, as compared to other groups. The results of our study suggested that topical curcumin application has neuroprotective effect in the healing tissue of diabetic wounded rats.

Key words: Diabetic rats, Wounds, Curcumin, GAP-43, Neuroprotective

INTRODUCTION

Delay in healing may lead to severe complications requiring extended hospitalization, amputation or even death of a patient. All over the world efforts are being made to identify the mechanisms lying behind delayed healing, so that new therapies can be developed. The literature indicates that in diabetic rat wound, delayed healing could be a consequence of less or delayed expression of different growth factors. Neovascularogenesis is vital for wound healing, as it plays necessary role in the delivery of oxygen, nutrients and other mediators at wound site. The process gets compromised in patients with diabetes, which consequently delayed wound healing (Scott, 2013).

Risk factors implicated in the development of diabetic foot ulcers are diabetic neuropathy, peripheral vascular disease, cigarette smoking, poor glycemic control, previous foot ulcerations or amputations, diabetic nephropathy, and ischemia of small and large blood vessels (Scott, 2013; Wu *et al.*, 2007). Diabetic patients often suffer from diabetic neuropathy due to several metabolic and neurovascular factors. There are many pathways for the development of a diabetic foot ulcer. In general, they include a combination of lower-limb arterial insufficiency, lower-limb diabetic neuropathy, and local trauma (Reiber *et al.*, 1999). About 20% of diabetic patients with foot ulcers will primarily have inadequate arterial blood flow, <50% will primarily have diabetic neuropathy, and <30% will be afflicted with both conditions (Reiber *et al.*, 1999). Poor circulation also damages peripheral nerves, causing a condition known as diabetic neuropathy or the loss of sensation in the arms and legs. The combination of poor blood flow and diabetic neuropathy complicates wound recovery.

Several studies in recent years have demonstrated curcumin as an antioxidant, and anti-inflammatory agent (Kloesch *et al.,* 2013; Kant *et al.,* 2014). Although some studies, including our studies, have reported the wound healing potential of curcumin (Mani *et al.,* 2002; Kant *et al.,* 2014, 2015). We have also shown that the modulation of different cytokines, growth factors and other enzymes at diabetic wound site by topical treatment of curcumin was responsible for its the antioxidant, anti-inflammatory and angiogenic potentials, which resulted in the acceleration of diabetic wound healing. However, to the best of our knowledge, detailed time-dependent study of curcumin on the neuronal regeneration in diabetic rats is lacking. Hence, in view of delayed wound healing in diabetic wounds due to neuropathy, and beneficial effects of curcumin, the present study was conducted to investigate the temporal effect of curcumin on the neuronal regeneration

at wound site in diabetic rats.

MATERIALS AND METHODS

Animals used and wound creation

Healthy adult male Wistar rats (170–200 g) procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (U.P.), India and were housed in standard polycarbonate cages. *Ad libitum* access to feed and water was provided and were maintained on a 12:12-hr light:dark cycle. Diabetes was induced by single intraperitoneal injection of streptozotocin (60 mg kg^{-1}; Sigma-Aldrich, USA). After 48 hours, animals with blood glucose levels e"300mg/dl were selected. Following 7 days of diabetes induction, the open excision-type wound 2×2 cm^2 (H"400 mm^2) was created, on the back of sixty diabetic animals under pentobarbitone sodium (40 mg kg^{-1}) anesthesia. After recovery from anesthesia, rats were housed individually in properly disinfected cages. The experimental protocol was approved and sanctioned by the Institute Animal Ethics Committee.

Groups

Wounded rats were divided equally in the following three groups:

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

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

III) Curcumin-treated: 400 µl of Curcumin (0.3%, Sigma Aldrich**)** in PF-127 gel (25%) was applied topically on wounds once daily for 19 days.

Collection of tissue and immunohistochemistry for growth associated protein-43 (GAP-43)

On days 3, 7, 14 and 19, five animals from each group were killed with an overdose of diethyl ether to collect granulation/healing tissue which was immediately preserved in 10% neutral buffer formalin and were embedded in paraffin. The 5 µm thick tissue sections were taken on 3-amino propyl triethoxysilane (APES) pre-coated slides for immunohistochemistry for GAP-43. Sections were immunostained for GAP-43 for axonal regeneration by incubating them with primary mouse monoclonal antibody (Novus Biologicals, Littleton, CO, USA) and biotinylated-conjugated goat anti mouse IgG (Novus Biologicals, Littleton, CO, USA). The sections were then incubated with extravidin-horse reddish peroxidase (HRP) (Sigma-Aldrich, USA). The aminoethylcarbazole (AEC) was used as chromogen substrate (AEC Staining Kit; Sigma-Aldrich, USA). Microphotographs were captured (Leica DFC450C) and thirty fields were observed (40x) for the quantification of GAP-43 positive nerve fibers.

Statistical analysis

All data are expressed as mean ± SEM. The 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 $P < 0.05$ or lower.

RESULTS AND DISCUSSION

The representative pictures of GAP-43 positive nerve fibers in the wound sections are presented in Figure 1 (40x). The distribution of the positive fibers was more concentrated in the blood vessels and hypodermis. The GAP-43 positive fibers were more in curcumin-treated group and significantly higher number per high power field was observed on day 14, as compared to other groups (Figure 2).

GAP-43 is a membrane protein that is expressed at high levels during neural development, and is newly produced in injured and regenerating adult nerve tissue. It is considered to be a marker for sprouting, and is usually associated with physiological events such as neuronal growth and synaptic plasticity (Verze *et al.,* 1999; Rage *et al.,* 2010). In the adult central nervous system, GAP-43 is present in several types of neurons and in regions of intense synaptic remodeling (Benowitz *et al.,* 1988; Benowitz *et al.,* 1990). In the normal adult peripheral nervous system, it is expressed at low levels in motor neuron axons (Li and Dahlstrom, 1993), but is highly expressed in sensory nerve fibers (Verze *et al.,* 1999). The expression of GAP-43 immunoreactive fibers decreases in diabetic patients (Bursova *et al.,* 2012).

In our study, curcumin markedly increased the expression of GAP-43 positive nerve fibers and revealed better sprouting and development of the newly

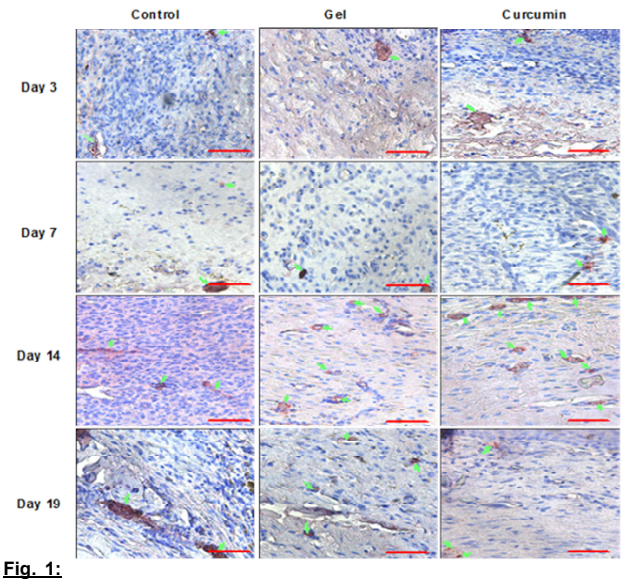

Fig. 1: Representative immunohistochemical GAP-43 staining of wound sections of control, gel- and curcumin-treated diabetic rats on days 3, 7, 14 and 19 post wounding (40x magnification and scale bar 40 µm). Arrow indicates the positive reaction for GAP-43 on the nerve fibers.

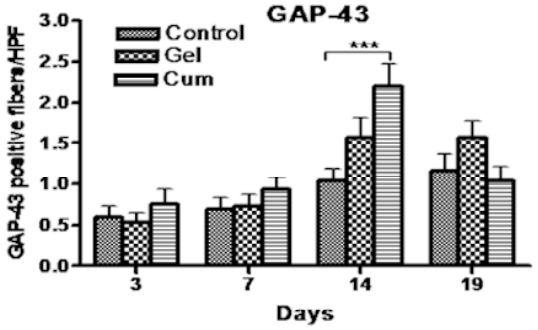

Fig. 2:
Semiquantitative analysis of GAP-43 positive nerve fibers at wound site in control. gel- and curcumin (Cum)-treated diabetic rats on days 3, 7, 14 and 19 post-wounding on days 3, 7, 14 and 19 post wounding. It was assessed by counting the number of GAP-43 positive nerve fibers in 30 random high-power fields (HPFS) (40x) in wounded dermis and hypodermis. Data are expressed as mean ± SE. * P<0.05, ** P<0.01 and *** P<0.001 vs. other group (s) on the same day. (n = 30)

regenerating axons at wound site in diabetic rats. Recent report has shown the role of curcumin in neurite outgrowth by increasing the expression of GAP-43 (Liao *et al.,* 2012). The results of our study suggested that topical curcumin application has neuroprotective effect in the healing tissue of diabetic wounded rats. From this study, it may be concluded that topical curcumin treatment possesses neuroprotective potential at diabetic wound site and this might be having role in the acceleration of wound healing in diabetic rats in our earlier studies.

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Research Article

PHARMACOKINETICS OF ENROFLOXACIN AND ITS METABOLITE CIPROFLOXACIN IN HEALTHY BUFFALO CALVES

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ABSTRACT

In the present study, pharmacokinetics and testing of dosage regimen was carried out in four healthy female buffalo calves after its i.v. administration @ 4 mg.kg^{-1}. Estimation of enrofloxacin and its active metabolite ciprofloxacin were done by using High Performance Liquid Chromatography (HPLC). The study revealed that enrofloxacin followed 2-compartment open model while ciprofloxacin followed non-compartment model. Mean ± S.E.M. distribution half life (t1/2 β), elimination half life (t1/2 β), mean residential time (MRT), volume distribution at steady state (VdSS) and total body clearance (ClB) of 0.28 ± 0.05 h, 2.95 ± 0.52 h, 2.91 ± 1.90 h, 5.38 ± 1.34 L.kg^{-1} and 34.58 ± 6.79 ml.kg^{-1}.min^{-1} were obtained for enrofloxacin while t1/2 β, MRT, VdSS and ClB of 2.79 ± 0.28 h, 3.60 ± 0.58 h, 13.19 ± 1.49 L.kg^{-1} and 68.85 ± 16.03 ml.kg^{-1}. min^{-1} were noted for ciprofloxacin. A % conversion of 51.86 ± 16.03 of enrofloxacin to ciprofloxacin occurred. Combined minimum therapeutic concentration of enrofloxacin + ciprofloxacin (Cp"min = minimum therapeutic concentration) of d" 0.25 µg.ml^{-1} was maintained from 0.048 - 6 h. Calculated dosage of enrofloxacin + ciprofloxacin for repetitive administration revealed a loading dose (D*) of 5.05 ± 0.65 mg.kg^{-1} and maintenance dose (D0) of 4.06 mg.kg^{-1} was calculated in the present study.

Key words: Kinetics, enrofloxacin, ciprofloxacin, testing of dosage regimen

INTRODUCTION

Enrofloxacin, a member of flouroquinolones was introduced by Bayer Pharmaceuticals in Germany in the year 1983. It is exclusively used in veterinary practice due to its wide spectrum of activity against many microbes apart from its toxicity. It is well tolerated in animals and used in small and large animals including cattle, buffalo, goat, sheep, horse etc. (Papich and Riviere, 2009). The toxicity is due to increase in Creatinine Phospho Kinase (CPK). It was demonstrated that acute toxicity by intravenous route (i.v) at 5 times the therapeutic dose in goat (15 mg.kg^{-1}) for 5 days and sub acute dose (15 mg.kg^{-1}) for 15 days intramuscularly (i.m) caused only temporary increase of CPK initially but returned to its normal level (Anuradha Kumari, 2004). Enrofloxacin is de-ethylated to ciprofloxacin which is used both in human and veterinary practice. The metabolite has been found to have a lower MIC requirement against certain gram negative microorganisms which is around 0.01 to 2.0 µg.ml^{-1} (Prescott and Yeilding, 1990). Besides, ciprofloxacin is active against mycobacterium species, against which enrofloxacin has its own activity (Prescott and Yeilding, 1990). Pharmacokinetic studies of enrofloxacin were carried out in lactating cows (Kaartinen *et al.,* 1995), she buffalo calves (Nitesh Kumar *et al.* 2003), sheep (Mengozzi *et al.,* 1996), female goats (Rao *et al.,* 2001; Uday Kumar *et al.* 2002; Narayan *et al.,* 2009) and horses (Otero *et al.,* 2009). Pharmacokinetics and testing of dosage regimen of cephazolin (Jayachandran *et al.,* 2014) and amikacin (Nirbhay Kumar *et al.,* 2013) in buffalo calves were carried out. So far, little work has been done on pharmacokinetics and specially on testing of dosage regimen of enrofloxacin in buffalo calves. Hence, the present study has been carried out.

MATERIALS AND METHODS

Experimental animals and route of administration

Four healthy female buffalo calves 1 to 1.5 years of age weighing between 150 to 180 kg of body weight were used in the present study. The animals were maintained on chopped wheat fodder, cattle feed cake, greens *etc*. with grazing for about 4-6 hours during experimental period. In each healthy buffalo calf, enrofloxacin (Enrocin 10% v/v) of Ranbaxy India Ltd., India was injected i.v. @ 4 mg.kg^{-1} in left jugular vein.

Collection of biological samples

Samples of plasma were collected at different time intervals *i.e.* 0.083, 0.167, 0.25, 0.333, 0.50, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h initially from right jugular vein and then alternately form both left and right jugular vein in heparinised centrifuge tubes. Samples were kept under deep freeze at - 20^{0}C and were analyzed within 48 h. After conducting studies in healthy animals, testing of dosage regimen was conducted based on dosage already calculated in healthy animals and

repeated the dosage regimen at particular dosage interval (γ) to verify whether minimum therapeutic concentration ($Cp^{"}$ min) was maintained or not?

Estimation of enrofloxacin and its active metabolite ciprofloxacin

Estimation of enrofloxacin and its active metabolite ciprofloxacin were done simultaneously by High Performance Liquid Chromatography (HPLC) method as described by Kung *et al.* (1993) and Nielsen and Gyrd-Hansen (1997) with slight modifications. Samples of standards of various strengths of plasma and test plasma samples collected at various time intervals were injected simultaneously to estimate the strengths of plasma samples. From standard graph of various strengths, the strengths of test samples were obtained. Limit of detection (LOD) is around 0.01d" $\mu g.ml^{-1}$.

Calculation of kinetic parameters and computation of dosage regimen

Kinetic parameters were obtained from the formulae derived by Gibaldi and Perrier (2007). For calculation of dosage regimen both enrofloxacin and its active metabolite ciprofloxacin were included. For calculation of D* (priming or loading dose) and D0 (maintenance dose) the following formulae of Baggot (1977) were used *i.e.* $D^* = Cp^{"}$(min). Vdarea ($e^{\beta \cdot \gamma}$), D0 = $Cp^{"}$ (min). Vdarea ($e^{\beta \cdot \gamma}$-1) where e is the base of natural logarithm, β is the elimination rate constant, γ is the dosage interval and $Cp^{"}$ (min) is the MIC of the drug.

Statistical analysis

The pharmacokinetic parameters were calculated by using students '*t*' test and the data are presented as Mean ± SEM (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

Fig.-1 shows the maximum concentrations in plasma Cp^{max} ($\mu g.ml^{-1}$) of enrofloxacin, ciprofloxacin and the combined concentration of both the drugs when enrofloxcin was administered @ 4 $mg.kg^{-1}$ in female buffalo calves. Mean ± SEM Cp^{max} ($\mu g.ml^{-1}$) of 4.17 ± 0.05 of enrofloxacin, 0.16 ± 0.02 of ciprofloxacin and combined concentrations of both the drugs of 4.33 ± 0.03 were obtained immediately at 0.042 h. Enrofloxacin and enrofloxacin + ciprofloxacin together followed 2-compartment model where as ciprofloxacin followed non-compartment model. Prescott and Yeilding (1990) and Mevious *et al.* (1990) noted that the drugs *viz.,* enrofloxacin and ciprofloxacin are effective around 0.01 to 2.0 $\mu g.ml^{-1}$ for different types of microorganism (Mevious *et al.,* 1990). In the present study 0.25 $\mu g.ml^{-1}$ is taken as therapeutic concentration in plasma (Cp^{ther}min). Cp^{ther}min of 0.25 $\mu g.ml^{-1}$ of enrofloxacin, ciprofloxacin and enrofloxacin + ciprofloxacin in female buffalo calves were maintained from 0.042 to > 5 h, 0.042 to > 0.0.333 h and 0.042 - 6 h., respectively.

Table 1 shows the kinetic parameters of enrofloxacin and its active metabolite ciprofloxacin after its i.v administration @ 4 $mg.kg^{-1}$ in healthy female buffalo calves. Distribution half life (t1/2 α) 0.28 ± 0.05 h denotes that enrofloxacin is moderately faster distributed to various organs and tissues in the body of female healthy buffalo calves. Similar t1/2 α of 0.28 ± 0.04 h in female healthy buffalo calves when 5 $mg.kg^{-1}$ i.v. administration of enrofloxacin (Nitesh Kumar *et al.,* 2003) and also similar to t1/2 α of 0.262 ± 0.099 h in buffalo bull calves (Verma *et al.,* 1999) were observed. A slightly longer t1/2 α of 0.60 ± 0.03 h in lactating goat was observed when 5 $mg.kg^{-1}$ i.v. was administered (Sudha Kumari *et al.,* 2004). A t1/2 α of 0.42 ± 0.05 h was noted in lactating goat (Udhay Kumar *et al.,* 2002) when 5 $mg.kg^{-1}$ i.v was administered. In case of horse, t1/2 α of 0.60 to 0.68 was reported (Giguere *et al.,* 1996). In pigs, similar t1/2 α of 0.23 ± 0.05 was reported (Anadon *et al.* 1999). In sheep a lower mean t1/2 α of 0.06 h was reported (Otero *et al.,* 2009).

Elimination half life (t1/2 β) of 2.40 ± 0.33 h in the present study in buffalo calves is more or less similar to 2.95 ± 0.52 h noted in female buffalo calves (Nitesh Kumar *et al.* 2003) and slightly lower which is 1.97 ± 0.23 h in buffalo bulls (Verma *et al.,* 1999). In case of goats, t1/2 β of 2.82 ± 0.33 h (Sudha Kumari *et al.,* 2004), 1.423 ± 0.15 h (Narayan *et al.,* 2009) and lower t1/2 β of 0.74 h after i.m. administration @ 2.5 $mg.kg^{-1}$ in goat (Rao *et al.,* 2001) were reported. On the other hand, in cattle a lower t1/2 β of 1.68 h in cows (Kaartinen *et al.,* 1995) was observed after its i.v. administration @ 5 $mg.kg^{-1}$. In horse, t1/2 β of 5.94 to 6.09 h was noted after its i.v administration @ 2.5 $mg.kg^{-1}$ (Giguere *et al.,* 1996) while t1/2 β of 4.4 h was noted in horse (Kaartinen *et al.*, 1997). In pigs, higher t1/2 β of 9.64 ± 1.49 h was noted (Anadon *et al.* 1999). In sheep, t1/2 β of 3.73 ± 0.44 h (Mengozzi *et al.* 1996), 3.73 ± 0.44 (Haritova *et al.* 2003) and 4.75 h (Otero *et al.,* 2009) were noted after i.v. administration of enrofloxacin.

Mean Residential Time (MRT) of 2.91 ± 1.90 h was noted in the present study while similar MRT of 3.05 ± 0.20 was noted in female buffalo calves (Nitesh Kumar *et al.,* 2003). In goats, similar MRT of 2.42 ± 0.19 h (Sudha Kumari *et al.,* 2004), 2.80 ± 0.24 h (Udhay Kumar *et al.,* 2002) and 2.42 ± 0.19 h (Narayan *et al.,* 2009) were noted while lower MRT of 1.54 h was noted in goat (Rao *et al.,* 2001). In sheep, also similar MRT of 3.02 ± 0.14 h was noted (Otero *et al.,* 2009). In horse, also similar MRT of 2.91 ± 0.17 was noted (Kaartinen *et al.,* 1997). On the other hand, a higher MRT of 12.77 ± 2.15 h was noted in pigs (Anadon *et al.,* 1999).

Vdarea of 7.47 ± 0.69 $L.kg^{-1}$ noted in the present study in female buffalo calves is similar to that of 7.92 ± 0.68 $L.kg^{-1}$ noted in female buffalo calves (Nitesh Kumar *et al.,* 2003) whereas a lower Vdarea of 0.61 ± 0.03

L.kg^{-1} was noted in male buffalo calves (Verma *et al.* 1999). Vdarea of 5.26 ± 1.23 L.kg^{-1} (Narayan *et al.,* 2009) and lesser Vdarea of 2.34 ± 0.54 L.kg^{-1} (Sudha Kumari *et al.* 2004) and 1.42 L.kg^{-1} in goats (Rao *et al.,* 2001) was observed. Vdarea > 1 L.kg^{-1} denotes that the drug apart from its well distribution, it is excreted through various routes (Baggot, 1977). It is excreted to a great extent *via* urine and also through bile also in many species of animals (Papich and Riviere 2009). High concentrations of enrofloxacin *i.e.* greater than plasma were found in milk and urine of lactating goats after s.c administration of the drug @ 5 mg.kg^{-1} (Narayan *et al.,* 2009) and i.v. administration @ 5 mg.kg^{-1} (Sudha Kumari *et al.,* 2004). Vdarea of 1.63 L.kg^{-1} in lactating cow observed after i.v. administration of enrofloxacin @ 5 mg.kg^{-1} (Kaartinen *et.al*., 1995). Vdarea of 0.77 ± 0.11 to 1.22 ± 0.07 L.kg^{-1} (Giguere *et al.* 1996)[23] and 2.3 L.kg^{-1} (Kaartinen *et al.* 1997) in horses were noted. In sheep Vdarea of 3.73 L.kg^{-1} after s.c. administration of 5 mg.kg^{-1} (Mengozzi *et al.,* 1996) and VdSS of 1.10 L.kg^{-1} after i.v. administration @ 5 mg.kg^{-1} (Otero *et al.,* 2009) were noted.

Total body clearance (ClB) value of 34.58 ± 6.79 ml.kg^{-1}.min^{-1} was observed in the present study in female buffalo calves. A similar value 32.40 ± 5.69 ml.kg^{-1}.min^{-1} was noted in female buffalo calves (Nitesh Kumar *et al.,* 2003) while a lower ClB value of 210.2 ± 6.79 ml.kg^{-1}.h^{-1} (3.52 ± 0. 031 ml.kg^{-1}.min^{-1}) was observed in male buffalo bulls (Verma *et al.* 1999). Lower ClB values of 1.50 ± 2.33 ml.kg^{-1}.min^{-1} (Giguere *et al.,* 1996), 4.83 ml.kg^{-1}.min^{-1} in horse 9.40 ± 0.54 ml.kg^{-1}.min^{-1} (Sudha Kumari *et al.,* 2004), 9.55 ±0.76 ml.kg^{-1}.min^{-1} (Udhay Kumar *et al.* 2002) and a high ClB value of 43.03 ± 9.10 ml.kg^{-1}.min^{-1} (Narayan *et al.,* 2009), 22.15 ml.kg^{-1}.min^{-1} (Rao *et al.,* 2001) in goats were noted after its i.m. administration. In sheep, low ClB values of 9.17 ± 2.4 ml.kg^{-1}.min^{-1} (Mengozzi *et al.,* 1996) and 4.83 ml. kg^{-1}.min^{-1} (Otero *et al.,* 2009) were noted.

Percent conversion of enrofloxacin ciprofloxacin (% enro to cipro) in the present study was observed to be 51.88 ± 16.03 whereas in female buffalo calves % enro to cipro was noted to be 46.95 ± 5.64 (Nitesh Kumar *et al.,* 2003). On the other hand, a little lower % enro to cipro of 35 was noted in goats (Rao *et al.,* 2001).

Kinetic parameters of ciprofloxacin were noted in Table -1. In the present study t1/2 β of 2.79 ± 0.28 h was noted in healthy female buffalo calves. Similar t1/2 β of 2.40 ± 0.33 h was noted in female buffalo calves (Nitesh Kumar *et al.* 2003). However, a lower t1/2 β of 0.934 h in cows was noted after i.v. administration of enrofloxacin (Gardofer, 1991). A t1/2 β of 1.38 h was noted for ciprofloxacin when enrofloxacin was given by i.m. route in goats (Rao *et al.,* 2001). However, a higher t1/2 β of 4.71 ± 0.67 h for ciprofloxacin after its single i.v. administration was noted (Singh *et al.,* 2001). MRT of 3.47 ± 0.47 h was noted in the present study for female buffalo calves (Table 1). More or less similar MRT of 3.47 ± 0.47 h was noted in female buffalo calves after its i.v. administration (Nitesh Kumar *et al.* 2003). However, lower MRT of 2.73 h was observed in goat (Rao *et al.,* 2001). Vdarea of 4.22 ± 0.51 L.kg^{-1} was noted for ciprofloxacin post i.v. administration @ 4 mg.kg^{-1} in goat (Singh *et al.,* 2001). Mean ClB value of 1329 L.kg^{-1}.h^{-1} (22.15 ml. kg^{-1}.min^{-1}) was noted in goats (Rao *et al.,* 2001) while lower ClB value of 11.19 ± 1.55 ml. kg^{-1}.min^{-1} in goats was noted (Singh *et al.,* 2001).

Variations among species, breed, sex, age and different methods of estimating the kinetic parameters may contribute the wide discrepancies in kinetic parameters reported by various workers (Jayachandran *et al.,* 1990; Baggot, 2001). It is well established that the clinical effectiveness of bactericidal drugs, specifically fluoroquinolones is mainly influenced by the height of the peak concentration relative to MIC (Cmax : MIC ratio) and maximum activity is achieved in the range 5-10 times of the MIC (Baggot, 2001). From Fig. 1, Cmax of combined concentration of enrofloxacin and ciprofloxacin is >5 µg.ml^{-1} while 0.25 is considered as MIC in the present study. Thus, Cmax : MIC ratio is more than 10 times and hence the drug is highly useful in combating the susceptible microbial infection in this species.

Table 3 shows the dosage regimen of combined concentration of enrofloxacin and ciprofloxacin to maintain MIC of 0.25 µg.ml^{-1} for the dosage interval (γ) of 8 h. Base on this a mean loading or priming dose

Table: 1

Comparison of kinetic parameters of enrofloxacin and ciprofloxacin when enrofloxacin was given i.v (4 mg.kg^{-1}) in healthy female buffalo calves. Mean ± SEM(n = 4)

Parameters(Unit)	Enrofloxacin	Ciprofloxacin
A (µg.ml^{-1})	1.78 ± 0.70	
B (µg.ml^{-1})	0.35 ± 0.04	
Cp^0 (µg.ml^{-1})	2.13 ± 0.68	
α (h^{-1})	2.71 ± 0.53	
t1/2 α (h)	0.28 ± 0.05	
β (h^{-1})	0.26 ± 0.04	0.31 ± 0.06
t1/2 β (h)	2.95 ± 0.52	2.79 ± 0.28
AUC 0 to " (mg/L.h)	2.25 ± 0.56	1.19 ± 0.33
AUMC (mg/L.h^{-1})	6.75 ± 1.96	4.79 ± 1.90
MRT (h)	2.91 ± 1.90	3.60 ± 0.58
Vd area (L.kg^{-1})	7.92 ± 0.68	
Vd SS (L.kg^{-1})	5.38 ± 1.34	13.19 ± 1.49
ClB (ml.kg.$^{-1}$min^{-1})	34.58 ± 6.79	68.85± 16.03
% conversion of enrofloxacin to ciprofloxacin		51.88 ± 16.03

All data are non-significant

A = Zero time conc. during distribution phase; B = Zero time conc. during elimination phase; Cp^0 = A + B; α = distribution rate constant; t1/2 α = distribution half life; β = elimination rate constant; t1/2 β = elimination half life; AUC0 to " = area under plasma conc. curve from zero time to infinite; AUMC = area under first moment curve; MRT = mean residential time; Vdarea = volume distribution based on area under curve; VdSS = volume distribution at steady state; ClB = total body clearance.

Table: 2
Kinetic parameters of enrofloxacin + ciprofloxacin together needed for calculation of dosage regimen of enrofloxacin in female buffalo claves.

PARAMETERS(UNIT)	MEAN ± SEM(n = 4)
A (μg.ml^{-1})	1.79 ± 0.81
B (μg.ml^{-1})	0.61 ± 0.05
α (h^{-1})	2.52 ± 0.53
t 1/2 α (h)	0.31 ± 0.05
β (h^{-1})	0.26 ± 0.05
t1/2 β (h)	3.04 ± 0.67
AUC (mg.L^{-1}.h)	3.54 ± 1.00
Vd area (L.kg^{-1})	5.20 ± 0.33

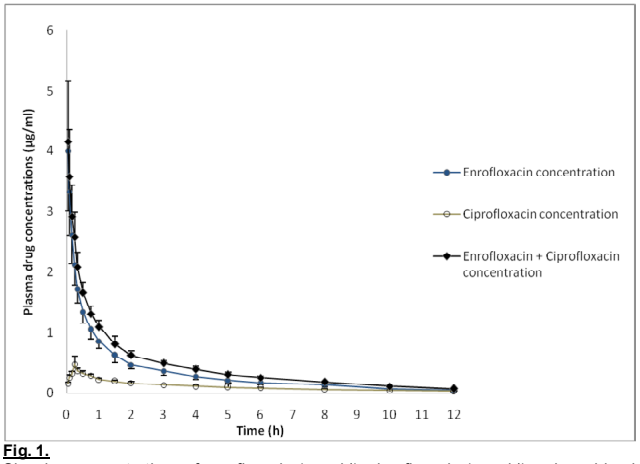

Fig. 1.
Showing concentrations of enrofloxacin (μg.ml^{-1}), ciprofloxacin (μg.ml^{-1}) and combined concentrations of enrofloxacin and ciprofloxacin (μg.ml^{-1}) at various time intervals when enrofloxacin was administered @ 4 mg.kg^{-1} in healthy female buffalo calves.

Table: 3
Calculated dosage regimen of enrofloxacin (enrofloxacin + ciprofloxacin together) used for repetitive administration in maintaining Cp$^{"}$ min (MIC) of 0.25 μg.ml^{-1} for the dosage interval (γ) of 8 h (n=4)

	Mean ± SEM
Loading dose (D*) in mg/kg	5.05 ± 0.65
Maintenance (D0) in mg/kg	4.06 ± 0.54

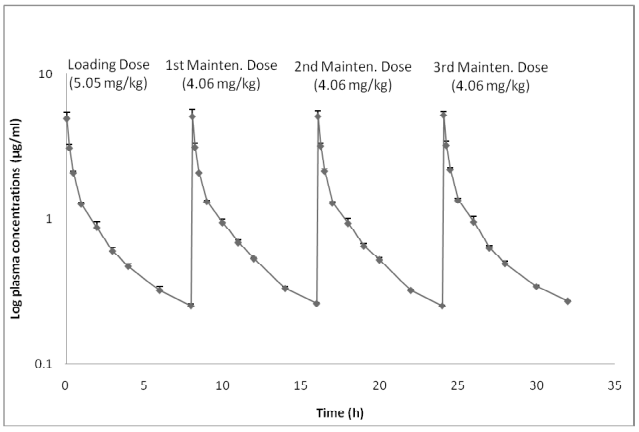

Fig. 2.
Testing of dosage regimen of enrofloxacin based on kinetic parameters of combined concentration of enrofloxacin and ciprofloxacin together after i.v. administration of enrofloxacin @ 4 mg.kg^{-1} in female buffalo calves.

(D*) of 5.05 mg.kg^{-1} was administered followed by mean maintenance dose (D0) of 4.06 at γ of 8 h and maintenance dose was repeated at 8 h interval. Thus, 3 times the D0 was repeated (Fig. 2). It clearly shows that minimum MIC was maintained throughout the course of administration of the drug.

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Research Article

EVALUATION OF ANTIMICROBIAL ACTIVITY OF ZINC OXIDE NANOPARTICLES AGAINST *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*

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ABSTRACT

The present study was aimed to investigate the antibacterial potential of zinc oxide (ZnO) nanoparticles dispersed in dimethyl sulfoxide (DMSO). Nanoparticles were synthesized by co-precipitation method while the stable dispersions of ZnO nanoparticles in DMSO were prepared with the aid of sonication. The synthesized nanoparticles were characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). ZnO nanoparticles were evaluated for antibacterial activity against *S. aureus* and *E. coli.* The mean maximum zone of inhibition of ZnO NPs against *E. coli* and *S. aureus* at 125 µg/ml is 21.50 mm and 23.50 mm, respectively**.** The ZnO nanoparticles seemed more effective than gentamicin against *E. coli* and *S. aureus.* It might be concluded that ZnO nanoparticles have potent antibacterial potential so, they might be highly suitable for applications to reduce antibacterial resistance.

Key words: ZnO nanoparticles, *S. aureus*, *E. coli,* antibacterial activity.

INTRODUCTION

The combination of nanotechnology and biology affords the opportunity for the development of novel materials in the nanometer size range which can be useful for various applications in biological science and clinical medicine (McNeil, 2005; Lanone and Boczkowski, 2006; Groneberg *et al.,* 2006). Nanotechnology is also very helpful in diagnostics, drug delivery, sunscreens, antimicrobial bandages, disinfectant, a friendly manufacturing process that reduce waste products, as catalyst for greater efficiency in current manufacturing process by minimizing or eliminating the use of toxic materials, to reduce pollution (e.g. Water and air filters) etc. (Sobha *et al.,* 2010). The distinctive properties and usefulness of nanoparticles (NPs) arise from a variety of features, including the comparable size of nanoparticles and biomolecules e. g. proteins and nucleic acids. The chief aspects believed to cause properties of nanoparticles to differ from their bulk counterparts include an increase in relative surface area and quantum effects (Lanone and Boczkowski, 2006; Nel *et al.,* 2006). The altered properties of nanoparticles, and their similarity to naturally occurring biological molecules allow them to interact with biomolecules and potentially affect cellular responses. In past, various types of nanoparticles e.g., metal nanoparticles, metal oxide nanoparticles, and polymer nanoparticles have been synthesized. The metal oxide nanoparticles is one of the most versatile materials among these due to their diverse properties and functionalities, and zinc oxide (ZnO) nanoparticles is the most preferred one due to their own importance and vast area of applications, e.g., gas sensor, chemical sensor, bio-sensor, cosmetics, storage, optical and electrical devices, window materials for displays, solar cells, and drug-delivery (Baxter and Aydil, 2005; Song *et al.,* 2006). Nano scale particles have emerged as novel antimicrobial agents due to their high surface area to volume ratio and currently, this has created the interest amongst the scientists/ researchers due to the rising problem of microbial resistances against metal ions, antibiotics and the development of resistant strains.

Thus, nanotechnology and nanomedicine can offer a more targeted approach, which promises significant improvements in the global problem of antimicrobial resistance. The recent growth in the field has stimulated us to evaluate the antimicrobial potentials of ZnO nanoparticles. In this study, we prepared the ZnO NPs, and its different concentrations, dispersed in dimethyl sulfoxide (DMSO), were evaluated for its antimicrobial activities against against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) in comparison to standard antimicrobials i.e. tetracycline and gentamicin to determine whether these NPs have potential utility in novel biomedical applications seeking to eliminate the pathogenic bacteria from the healthy body tissues and environment.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were of analytical grade. Zinc acetate and sodium hydroxide were used for the preparation of ZnO nanoparticles, and were purchased from Sigma Aldrich. DMSO used as

base fluids for the dispersion of nanoparticles was purchased from SRL. *Escherichia coli* (MTCC 2127) and *Staphylococcus aureus* (MTCC 1430) bacterial strains were gifted from the Department of Biotechnology, Univerity of Jammu. Muller-Hinton Agar (MHA) and Muller-Hinton Broth (MHB) used in antibacterial studies were purchased from Himedia. The standard antibiotics i.e. tertracycline and gentamicin were purchased from Sigma Aldrich.

Synthesis and characterization of ZnO nanoparticles

The precursors used in the synthesis of ZnO nanoparticles were Zinc acetate [$Zn(CH_3COO)_2.2H_2O$] and sodium hydroxide. A 0.5M aqueous solution of zinc acetate was kept under constant stirring using magnetic stirrer at 80°C to completely dissolve for one hour. After complete dissolution of zinc acetate, 2.5M NaOH aqueous solution was added under high speed constant stirring, drop by drop (slowly for 45 min) touching the walls of the vessel till the pH reaches to 12. The reaction was allowed to proceed for 2 hrs after complete addition of sodium hydroxide. After the completion of reaction, the solution was allowed to settle for overnight and further, the supernatant solution was separated carefully. The remaining solution was centrifuged for 10 min, and the precipitate was removed. Thus, precipitated ZnO Nanoparticles were washed three times with triply distilled water and ethanol to remove the byproducts which were bound with the precipitate and then dried in oven at about 60°C. The white powder obtained is subjected to calcinations at 600°C for 3hours.

The synthesized nanoparticles were characterized by using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The size and morphology of nanoparticle was done by using Transmission Electron Microscope [Hitachi (H-7500) microscope] operating at 80 kV. The morphology of the particles was observed by a scanning electron microscope (SEM-EDS) using SEM (make JEOL Model JSM - 6390LV).

Antibacterial activity of ZnO nanoparticles

Assessment of zone of inhibition

Antibacterial activities of the synthesized ZnO nanoparticles were performed against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. Tertracycline and gentamicin were used as positive control. DMSO was used as negative control. Muller-Hinton Agar was used for the study and the nanoparticles as well as antibiotics were tested for their antibacterial activity by agar well diffusion method (Perez *et al.* 1990) In this method, each flask containing autoclaved Muller Hinton agar was seeded with 24 h culture of both bacterial strains (10^8 cfu/ml) and then 10 ml was poured onto 100 mm petri plates while still molten. A sterile cork borer was used to cut wells of 6 mm diameter in each of plates. Suitable dilutions of nanoparticles (0.12 to 125 µg/ml) and antibiotics were made with DMSO and 20µl of each was carefully placed in each well. The plates were incubated at 37°C for 24 h. The antibacterial activity was tested by measuring the diameter of the inhibition zone formed around the well. The zones of inhibition obtained were measured and all the samples were tested in triplicates. The activity index and fold increase were calculated from the values of zone of inhibition for the comparative analysis of ZnO nanoparticles with the standard antibiotics.

Assessment of activity index

The assessment of activity index was obtained by comparing the resultant zones of inhibition of nanoparticles with the standard reference antibiotic using the following formula:

$$\text{Activity Index (AI)} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

Assessment of fold increase

Increase in fold area was assessed by calculating the mean inhibition zone produced by the standard reference antibiotic and nanoparticles. The fold increase area was calculated by the equation:

Fold increase (%) = (b -a/a)100

where, a and b refer to the inhibition zones of antibiotic and sample, respectively.

RESULTS AND DISCUSSION

The TEM image of ZnO nanoparticles revealed that the product consisted of approximate spherical particles with the average size of 28-43 nm (Fig. 1A). It can be observed that ZnO nanoparticles mainly present in granules with approximate spherical shape and are well crystallized. The SEM micrograph of the ZnO nanoparticles indicated a homogeneous shape and size for ZnO nanoparticles and aggregations of chemically synthesized nanoparticles (Fig. 1B). Antibacterial activity of synthesized nanoparticles was evaluated against bacterial strains using standard zone of inhibition (ZOI) assay. The representative images of zone of inhibitions against *S. aureus* (Fig. 2A,B,C) and *E. coli* (Fig. 2D,E,F) by tetracycline, gentamicin and ZnO nanoparticles, respectively revealed that inhibition of bacterial growth increased with the increase in antibiotic/ nanoparticles concentration. The average zone of inhibitions in comparative form for standard antibiotics and ZnO nanoparticles against *S. aureus* and *E. coli* are presented in Table 1. The maximum zone of inhibition of ZnO nanoparticles against *E. coli* and *S. aureus* at 125 µg/ml is 21.50 mm and 23.50 mm, respectively (Table 1). Previous studies have reported increased bacterial growth inhibition with the increase in concentration of nanoparticles (Wahab *et al.* 2010).

The zone of inhibition in present study clearly

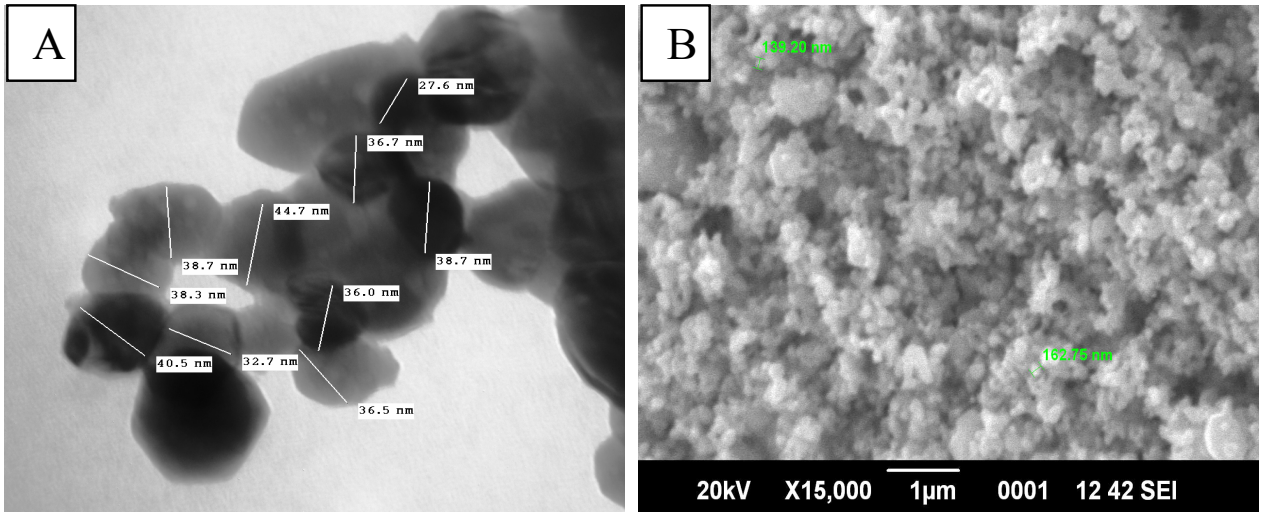

Fig. 1. Micrograph of TEM (A) and SEM (B) of ZnO nanoparticles

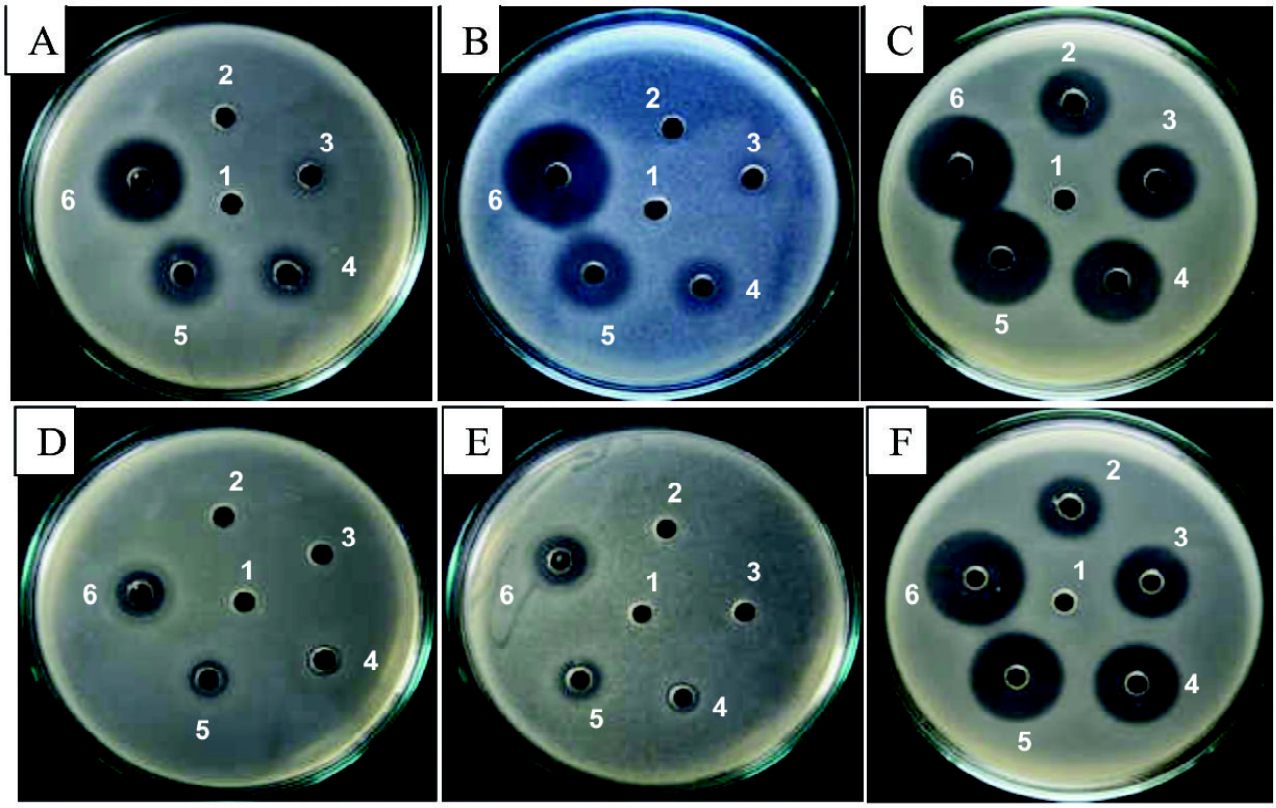

Fig 2. Antibacterial activity of Tetracycline (A & D), gentamicin (B & E) and ZnO nanoparticles (C & F) against *S. aureus* (A-C) and *E. coli* as (D-E) at different concentrations i.e. (1) 0.00 µg/ml, (2) 0.48 µg/ml, (3) 1.95 µg/ml, (4) 7.8 µg/ml, (5) 31.25 µg/ml and (6) 125 µg/ml.

indicated the biocidal action of ZnO nanoparticles and the smaller size of the nanoparticles might led to increased membrane permeability and cell destruction (Ankanna and Savithramma, 2011). Moreover, the large surface area of the nanoparticles may lead to their tightly adsorbtion on the surface of the bacterial cells, which disrupt the bacterial membrane and causes leakage of intracellular components which resulted in death of the bacterial cells (Qi *et al.* 2004). It was observed in the present study that the sensitivity of *S. aureus* was more than *E. coli* for both tetracycline and gentamicin at the same concentration. The sensitivity of tested bacteria against the action of ZnO nanoparticles was also similar to standard antimicrobials at the same concentration. This was might be due to the difference in the structure of the bacteria. These results are in concurrence with

the earlier studies in which ZnO nanoparticles showed more sensitivity against *S. aureus* than *E. coli* (Baek and An, 2011). The antibacterial action of ZnO nanoparticles may be due the cell membrane damage and production of ROS. The earlier studies have reported that membrane damage is caused by direct or electrostatic interaction between ZnO and surface of cell, cellular internalization of ZnO nanoparticles, and the production of reactive oxygen species such as H_2O_2 in cells (Stoimenov *et al.,* 2002). The antibacterial effects of ZnO nanoparticles were in concentration dependent manner between 0.12 to 125 µg/ml concentrations only. But, the concentration dependent pattern is not followed beyond 125 µg/ml concentration. Hence, the optimum concentration for ZnO nanoparticles is 125 µg/ml. This type of behavior was might be due to agglomeration of nanoparticles at higher concentrations. It has been suggested earlier that there is an optimum concentration which has higher effect compared to those concentrations that have more or less than the optimum (Vani *et al.* 2011). Thus, the effect of nanoparticles is not entirely dependent on increasing concentration of nanoparticles. It was also observed in present study that the standard antibiotics were not effective at lower concentrations i.e. below 3.9 µg/ml for tetracycline and 7.8 µg/ml for gentamicin against both tested bacteria (Table 1). However, ZnO nanoparticles were effective below these concentrations in present study (Tables 1). This revealed that the ZnO nanoparticles would provide antibacterial action for longer duration in comparison to tetracycline and gentamicin. So, these could be a choice of replacement for tetracycline and gentamicin in different areas to reduce the appearance of antimicrobial resistance.

The activity index (AI) is calculated to express the link among the zone of inhibition of the nanoparticles with the standard antibiotics (Selvarani and Prema, 2013). Value of AI greater than 1 reveals that tested compound possesses strong efficacy than standard antibiotic for a particular bacteria at same concentration. The results of AI for ZnO nanoparticles with respect to tetracycline and gentamicin against *S. aureus* and *E. coli* are summarized in Tables 2. The AI of ZnO Nanoparticles w.r.t. tetracyclineand gentamicin against both *E. coli* and *S. aureus* is also found higher than 1 at all the compared concentrations. It was observed for the ZnO nanoparticles that the highest AI against E. coli w.r.t. tetracycline was 2.28 at 3.9 µg/ml and w.r.t. gentamicin, it was 2.68 at 7.8 µg/ml. Further, it was recorded that the highest AI of ZnO nanoparticles against *S. aureus* w.r.t. tetracycline was 2.27 at 3.9 µg/ml and w.r.t. gentamicin, it was 2.52 at 7.8 µg/ml. The AI of ZnO nanoparticles was found more for gentamicin than tetracycline against *E. coli* and *S. aureus* at the same concentration. The calculation of percent fold increase is another method of determining the relative effect of tested compound in comparison to any standard antibiotic/drug. The positive value of fold increase

Table 1.
Zone of inhibition for tetracycline, gentamicin and ZnO nanoparticles against *S. aureus* and *E. coli* at different concentrations

Conc. (µg/ml)	Zone of inhibition (mm) against *S. aureus*			Zone of inhibition (mm) against *E. coli*		
	Tetracycline	Gentamicin	ZnO nanoparticles	Tetracycline	Gentamicin	ZnO nanoparticles
125	21.50	13.00	23.50	18.83	11.50	21.50
62.5	18.00	12.33	22.17	15.17	10.67	20.33
31.25	14.17	11.17	21.33	13.50	10.00	19.67
15.6	12.33	9.83	20.17	11.33	8.67	19.17
7.8	10.33	7.67	19.33	9.33	6.83	18.33
3.9	8.00	—	18.17	7.67	—	17.50
1.95	—	—	17.50	—	—	16.50
0.97	—	—	16.17	—	—	15.17
0.48	—	—	14.50	—	—	13.33
0.24	—	—	13.17	—	—	12.17
0.12	—	—	12.17	—	—	11.17

Table 2.
Activity index and fold increase (%) of ZnO nanoparticles with respect to (w.r.t.) tetracycline and gentamicin against *S. aureus* and *E. coli* at different concentrations

Conc.(µg/ml)	Activity Index w.r.t. Tetracycline		Activity Index w.r.t. Gentamicin		Fold increase (%) w.r.t. Tetracycline		Fold increase (%) w.r.t. Gentamicin	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
125	1.09	1.14	1.81	1.87	9.30	14.16	80.77	86.96
62.5	1.23	1.34	1.80	1.91	23.15	34.07	79.73	90.63
31.25	1.51	1.46	1.91	1.97	50.59	45.68	91.04	96.67
15.6	1.64	1.69	2.05	2.21	63.51	69.12	105.08	121.15
7.8	1.87	1.96	2.52	2.68	87.10	96.43	152.17	168.29
3.9	2.27	2.28	—	—	127.08	128.27	—	—

indicates the better effects of tested compound against standard drug. The values of fold increase of ZnO nanoparticles w.r.t. tetracycline and gentamicin against both *E. coli* and *S. aureus* are positive at all the compared concentrations of our study (Table 2). It was observed for the ZnO nanoparticles that the maximum fold increase against *E. coli* w.r.t. tetracycline was 128.27 % at 3.9 µg/ml and w.r.t. gentamicin, it was 168.29 % at 7.8 µg/ml (Table 2). Further, it was recorded that the maximum fold increase of ZnO nanoparticles against *S. aureus* w.r.t. tetracycline was 127.08 % at 3.9 µg/ml and w.r.t. gentamicin, it was 152.17 % at 7.8 µg/ml (Table 2). The fold increase of ZnO nanoparticles was found more for gentamicin than tetracycline against either *E. coli* or *S. aureus* at the same concentration. So, among the gentamicin and tetracycline, ZnO nanoparticles seems more effective than gentamicin. Inorganic antibacterial agents such as metals and metal oxides are advantageous in comparison to organic compounds due to their stability at elevated temperatures and pressures with no loss of medicinal property (Sondi and Sondi, 2004). As, the prevention of microbial infections can reduce the mortality and associated treatment of costs, thus coating of ZnO nanoparticles on large active surface area, on cellulose bandages, uniforms, bed linen, medical equipment etc. could make a considerable input in reducing bacterial contamination. The credible antibacterial activities of ZnO nanoparticles in present study makes the future studies interesting in evaluation of antibacterial potential of these nanoparticles against other antibiotic resistant bacteria as well as their *in vivo* efficacy.

Thus, it was observed from the results of present study that the ZnO nanoparticles seemed more effective than tetracycline and gentamicin, particularly at low concentrations, against gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria. In conclusion, its potent antibacterial potentials extends its application in medical field, water disinfection etc.

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Research Article

PROTECTIVE EFFECTS OF *AJUGA MACROSPERMA* ON TISSUE CHANGES IN CYCLOPHOSPHAMIDE TREATED RATS

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ABSTRACT

The present study was planned to evaluate the pathological changes following oral administration of methanolic extract of *Ajuga macrosperma* in rats treated with cyclophophamide (CP). Forty eight (48) Wistar rats of either sex were divided randomly into eight groups of 6 rats each viz. groups I, II, III, IV, V, VI, VII and VIII. Group I served as control. Group II rats were administered CP @ 100 mg/kg b wt, orally on 9th and 16th day, group III was given levamisole @ 50 mg/kg b wt orally for 28 days, group IV was administered CP @ 100 mg/kg b wt orally on 9th and 16th day and levamisole @ 50 mg/kg b wt orally daily for 28 days, Group V was given methanolic extract of *Ajuga macrosperma* (MEAM) i.e. MEAM-1 @ 100 mg/kg b wt orally for 28 days, group VI was given MEAM-2 @ 200 mg/kg bwt, orally for 28 days, group VII was administered MEAM-1 @ 100 mg/kg b wt orally for 28 days and CP @ 100 mg/kg b wt orally on 9th and 16th day, group VIII was given MEAM-2 @ 200 mg/kg bwt, orally for 28 days and CP @ 100 mg/kg b wt. Histopathological studies revealed severe congestion of large and small blood vessels, severe sinusoidal congestion leading to loss of sinusoidal spaces, Kupffer cell proliferation, accumulation of mononuclear cells around many congested blood vessels, severe degeneration and swelling of hepatocytes throughout the parenchyma in liver; severe depletion of lymphoid cells, reticular cell hyperplasia, and depletion of red pulp and presence of hemosidrin pigment in spleen; severe congestion of large blood vessels and interstitial hemorrhages, vacuolation of glomeruli, coagulative necrosis of varying degrees in many of the renal tubular epithelial cells and infiltration of mononuclear cells in the interstitium of kidney and presence of irregular and elongated neurons in brain were observed in cyclophosphamide treated rats which were ameliorated by treatment with MEAM in a dose dependent manner. It is concluded from this study that CP administration @ 100mg/kg b wt, orally produced tissue changes that were ameliorated by MEAM (100 and 200 mg/kg b wt, orally for 28 days) in rats.

Key words: *Ajuga macrosperma*, cyclophosphamide, pathology, rat

INTRODUCTION

Plant kingdom is considered as the key reservoirs of natural entities having tremendous medicinal value. Ayurveda and other traditional literature mention about 1500 plants with medicinal uses and around 800 of these have been used as ethnomedicine in the treatment of various human ailments. About two third of the Indian population substantially depends on the herbal based remedies for the treatment of various ailments. Unfortunately, a large number of plants with medicinal values have not yet been evaluated for their pharmacological properties or chemical constituents. Plants have been recognized since antiquity for recovery from various ailments without side effects. In view of an increasing need to develop new effective herbal remedies or drugs for health care; traditional medicinal plants have recently received adequate attention globally by pharmaceutical companies and Veterinary medical professionals. Herbal formulations are effective, relatively cheaper and safe alternative treatment for various diseases since most of the synthetic drugs available in the market provide only symptomatic relief and have untoward effects (Fulzele *et al.,* 2002). Thus, efforts are being made to discover newer drugs from diversified plant kingdom to develop and provide therapeutic cure which would be free from undesirable side effects as well as economical and easily acceptable by the veterinary and medical professionals throughout the world.

Immune system play vital role as defensive system against infectious agents causing diseases in man and animals. Immunomodulation is an effective means of altering the immune system in favour of the host either by stimulating the immune cells for better performance or by suppressing their response in case of auto-immune disorder and tissue transplantation. Activation of major immune cells including T and B lymphocytes by immunostimulatory agents helps to restore immune balance necessitating the discovery of such newer agents from synthetic or natural sources. Many plants have active principles which have immunomodulatory effects. There is a need for investigating the active principles of various plants responsible for immunomodulatory properties. These immunomodulatory agents are becoming very popular as natural health remedy, tend to boost immunity and protect from the invasion by pathogenic organism causing diseases in man and animals (Agrawal *et al.,* 2010). Herbal immunomodulators have been critically considered as an alternative and/or adjunct to conventional therapy against various infectious diseases. *Ajuga macrosperma* is such a plant which is known as an immunomodulator in the Ayurveda but as such no literature is present to on the immunomodulatory property of this plant. Keeping in view the above facts regarding

medicinal properties of the traditional plants and lack of information on immunomodulatory potential of *Ajuga macrosperma*, the present study was planned to evaluate histopathological changes following administration of methanolic extract of *Ajuga macrosperma* (MEAM) in CP treated rats.

MATERIALS AND METHODS

Forty eight (48) Wistar rats of 60 to 75 days of age, weighing around 175g, of either sex, obtained from Experimental Animal House of the College of Veterinary and Animal Sciences, GB Pant University of Agriculture and Technology, Pantnagar, were divided randomly into eight groups of 6 rats each as mentioned in the Table 1. The dose of methanolic extract of *Ajuga macrosperma* (MEAM) used was selected on the basis of LD_{50} that was calculated on the basis of a pilot experiment conducted before the start of the present study (Lone, 2016) and that of levamisole and CP was selected on the basis of available literature (Morais *et al.,* 1999; Chabner *et al.,* 2001).

The experiment was conducted for a period of 28 days. At 28th days post treatment (DPT), the rats were sacrificed humanely. Detailed post mortem examination of the sacrificed animals was done and the gross lesions were duly recorded. Tissue samples from of liver, spleen, kidney and brain tissues were collected and fixed in 10% buffered formalin. These samples were processed for histopathological examination following routine procedures (Luna, 1968).

RESULTS

Gross pathology

Liver: No gross lesions were observed in liver of groups I, III, V, VI and VIII rats. In group II rats, liver was reddish in colour and slightly increased in size. In groups IV and VII rats, liver was slightly pinkish.

Kidneys: Kidneys of groups II and VII were swollen, slightly reddish in coloration and congested. Kidneys of rats of other groups showed no significant changes.

Spleen: Spleen of group II rat were and reddish in colour. Spleen of all other groups did not reveal any untoward change.

Brain: No gross lesions were observed in the rats of any of the groups.

Histopathological examination

Liver: No lesions could be observed in the liver of groups I, III, IV, V and VI rats. Liver of group II rats showed severe congestion of large and small vessels, severe sinusoidal congestion leading to loss of sinusoidal spaces, Kupffer cell proliferation, and accumulation of mononuclear cells around many congested blood vessels, severe degeneration and swelling of hepatocytes throughout the parenchyma. Similar lesions but of varying intensity were seen in groups IV, VII, and VIII. Lesions in liver were severest in group II followed by groups VII, IV and VIII **(Plate 1)**. These findings suggest healing potential of MEAM in cyclophosphamide induced liver damage.

Spleen: No lesions were found in spleen of groups I, III, V, VI and VIII rats. Spleen of group II rats showed severe depletion of lymphoid cells, reticular cell hyperplasia,

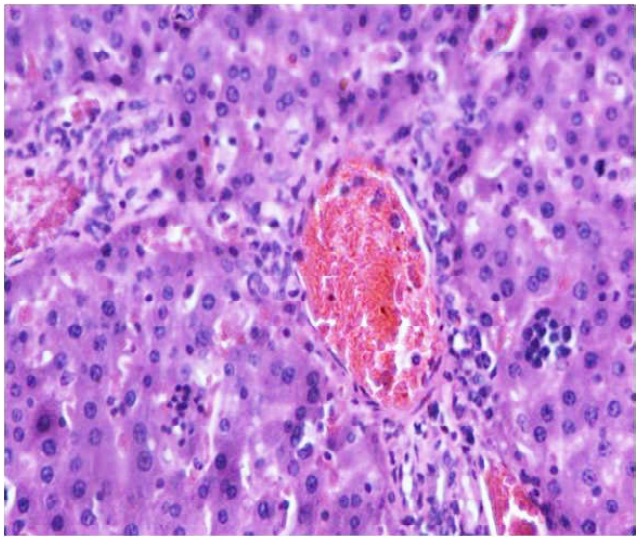

Plate 1:
Photomicrograph of liver showing severe congestion of large and small vessels, severe sinusoidal congestion leading to loss of sinusoidal spaces, Kupffer cell proliferation, accumulation of mononuclear cells around many congested blood vessels, severe degeneration and swelling of hepatocytes throughout the parenchyma. (Group II, H&E, 400X).

Table 1:
Experimental design in tabular form

Groups	Treatments	No. of rats
I.	1 ml distilled water orally	6
II.	CP @ 100 mg/kg b wt orally on 9th and 16th day of experiment	6
III.	Levamisole @ 50 mg/kg b wt, orally daily for 28 days	6
IV.	CP @ 100 mg/kg b wt. orally on 9th and 16th day of experiment +Levamisole @ 50 mg/kg b wt orally daily for 28 days	6
V.	MEAM @ 100 mg/kg b wt, orally daily for 28 days	6
VI.	MEAM @ 200 mg/kg b wt, orally daily for 28 days	6
VII.	CP @ 100 mg/kg b wt. orally on 9th and 16th day of experiment + MEAM @ 100 mg/kg b wt, orally daily for 28 day	6
VIII.	CP @ 100 mg/kg b wt, orally on 9th and 16th day of experiment + MEAM @ 200 mg/kg b wt, orally daily for 28 days	6

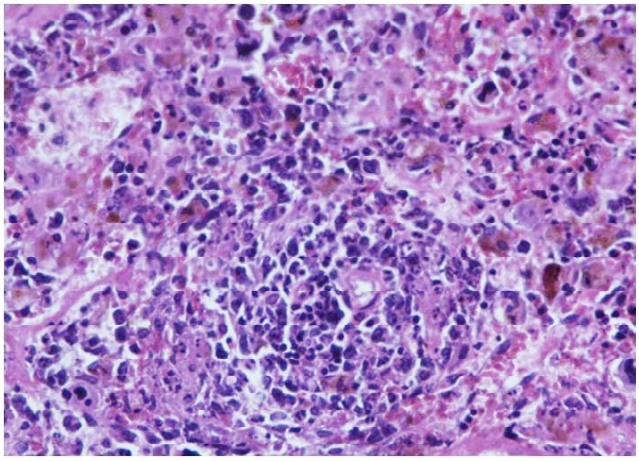

Plate 2:
Photomicrograph of spleen showing severe depletion of lymphoid cells, loss of red pulp and presence of hemosidrin pigment. (Group II, H&E, 400X).

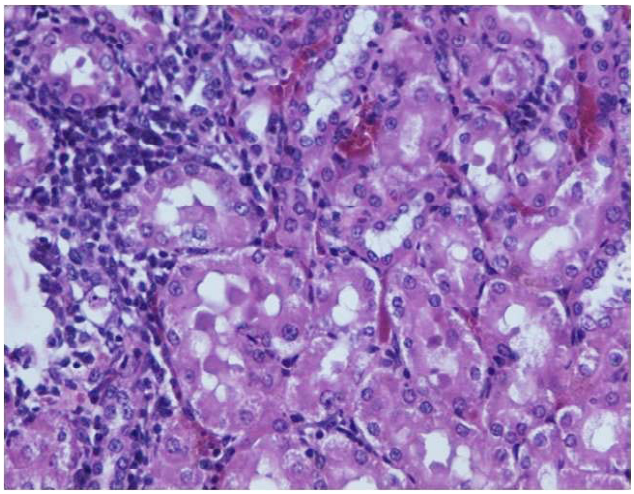

Plate 3:
Photomicrograph of kidney showing interstitial hemorrhages, coagulative necrosis of varying degrees in many of the kidney tubular epithelial cells in many of tubules and infiltration of mononuclear cells in interstitium. (Group II, H&E, 400X).

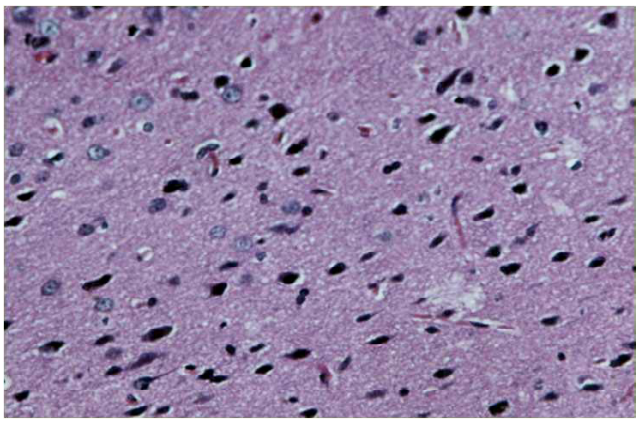

Plate 4:
Photomicrograph of brain showing showed presence of irregular and elongated neurons. (Group II, H&E, 400X).

and depletion of red pulp and presence of hemosiderin pigment. Lesions were similar and of varying intensity in groups IV, VII and VIII rats. Lesions in spleen were severest in group II followed by groups VII, IV and VIII rats **(Plate 2)** indicating the ameliorative effect of MEAM in spleen.

Kidneys: No lesions were evident in kidneys of groups I, III, V, and VI rats. Kidneys of group II rats revealed severe congestion of large blood vessels and interstitial hemorrhages, vacuolation of glomeruli, coagulative necrosis of varying degrees in many of the kidney tubular epithelial (KTE) cells in many of tubules and infiltration of mononuclear cells in interstitium. Lesions were similar and of varying intensity in groups IV, VII and VIII rats. Lesions in kidneys were severest in group II followed by groups VII, IV and VIII rats **(Plate 3)**. These results showed the healing potential of MEAM in cyclophosphamide induced kidney damage.

Brain: No lesions could be recorded in the brain of groups I, III, IV, V and VI rats. Brain of group II rats showed presence of irregular and elongated neurons. Lesions were similar and of varying intensity in groups IV, VII and VIII rats. Lesions in brain were severest in group II followed by groups VII, IV and VIII rats (Plate 4).

Thus, it is evident from these histopathological studies that MEAM has got ameliorative effect on cyclophosphamide induced liver, kidney, spleen and brain damage as it restores the architecture of these organs in a dose dependent manner.

DISCUSSION

Gross and histopathological changes were evident in CP treated rats whereas these changes were either of mild degree or were absent in MEAM treated rats which are indicative of protective effects of MEAM in CP induced pathological changes in liver, kidney, spleen and brain tissues. Cyclophosphamide, a prodrug which is metabolized by liver cytochrome P450 (CYP) enzymes to form an active metabolite 4-hydroxy cyclophosphamide and belongs to the class of antimetabolite drugs, is used in cancer chemotherapy and produces its action by attaching an alkyl group to at the number 7 nitrogen atom to the guanine base of the imidazole ring and interferes with the replication of DNA by producing intrastrand and interstrand DNA cross links which is irreversible and is responsible for cell apoptosis (Hall and Tilby, 1992; Emadi *et al.,* 2009). Thus, development of pathological lesions in the tissues of CP treated rats in the present study are attributed to this reason.

Liver of CP treated rats showed severe congestion of large and small vessels, severe sinusoidal congestion leading to loss of sinusoidal spaces, Kupffer cell proliferation, and accumulation

of mononuclear cells around many congested blood vessels, severe degeneration and swelling of hepatocytes throughout the parenchyma. Oral CP is rapidly absorbed and metabolized by mixed-function oxidase enzymes (cytochrome P450 system) in the liver to active metabolites (Cohen and Jao, 1970). These pathological effects are due to hepatoxic effects of CP as it increases hepatic microsomal enzyme activity which might have resulted in accelerated metabolism of the drug producing its active metabolites in excess, subsequently enhancing toxic effects on liver as well as other tissues of the body (Donelli *et al.,* 1976). Ameliorative effect of MEAM may be attributed to its antioxidant action which might have reduced the production of oxidative radicals as observed in the present study (Lone, 2016).

Kidneys of CP treated rats revealed severe congestion of large blood vessels and interstitial hemorrhages, vacuolation of glomeruli, coagulative necrosis of varying degrees in many of the kidney tubular epithelial (KTE) cells in many of tubules and infiltration of mononuclear cells in interstitium. The active metabolites of CP are also distributed to all tissues and are assumed to cross the placenta and also present in breast milk (Wiernik and Duncan, 1971). Cyclophosphamide metabolites are usually excreted in the urine in unchanged setting of renal dysfunction (Haubitz *et al.,* 2002) which is responsible for nephrotoxic effects of CP observed in the present study also. In addition, several studies indicate that CP has a pro-oxidant character and generation of oxidative stress after CP administration leads to decrease in the activities of antioxidant enzymes and increase in lipid per-oxidation in liver, lung and serum of mice and rats (Premkumar *et al.,* 2001).

Spleen of rats treated with CP showed severe depletion of lymphoid cells, reticular cell hyperplasia and depletion of red pulp and presence of hemosidrin pigment whereas such changes were of very low intensity or absent in MEAM treated rats. Cyclophosphamide cause immunosuppression by elimination of T regulatory cells in naive and tumour-bearing hosts, induction of T cell growth factors, such as type I IFNs, and/or enhanced grafting of adoptively transferred tumor-reactive effector T cells by the creation of an immunologic space niche (Sistigu *et al.,* 2011). The hematopoietic and lymphoid system is very susceptible to the effects of alkylating agents. Cessation of mitosis and disintegration of formed elements was observed within 8 hours after administration of the sub lethal dose of CP in the marrow and lymphoid tissues in humans (Calabresi and Chabner, 1991). Thus, pathological changes in spleen in the present study is due to susceptibility of spleen and other lymphoid organs to CP and MAEM revealed ameliorative effect by reversing this action due to its immunomodulatory potential (Lone, 2016).

Lesions in the brain tissues are also attributed to wide distribution of metabolites of CP in these tissues causing toxic effects due to DNA aberrations (Emadi *et al.,* 2009). In addition, CP causes teratogenecity, hepatotoxicity, anomalies of central nervous system and skeletal system in rats, mice, rabbits, monkeys and human beings and induces leucopoenia and thrombocytopenia which may be due to its physiochemical properties such as lipophilicity, capacity to cross biological membranes and stability in aqueous solutions. In patients treated with CP, lymphocytopenia and thrombocytopenia, was evident within 24 hours which lasted extended for many days and reduction in platelet and erythrocyte counts persisted for 3 weeks after therapy (Fisher *et al.,* 1993).

Oxidative radicals scavenging potential of MEAM was evident in this study which might have led to its ameliorative effect in CP induced toxic effects on various visceral organs including brain tissue. Hepataoprotective effect of MAEM observed in this study might have enhanced the elimination of drugs from the body leading to reduction in retention of drugs and its metabolites in the body tissues.

It is concluded from this study that the adverse effects of cyclophosphamide were ameliorated by MEAM @ 100 and 200 mg/kg b wt on tissue changes. Further, it was also concluded from the present study that MEAM-2 @ 200 mg/kg b. wt. was more effective in ameliorating the tissue changes produced by cyclophosphamide than MEAM-1 @ 100 mg/kg b. wt.

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Research Article

PHARMACOKINETICS OF AMOXICILLIN AND CLOXACILLIN FOLLOWING SINGLE DOSE INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION IN GOATS

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ABSTRACT

A commercial preparation of Amoxicillin and Cloxacillin (1:1), @ 10 mg/kg body weight was injected in goats by i.v. and i.m. routes of administration and the plasma time profile of amoxicillin and cloxacillin was studied. One and two-compartment model adequately described plasma-concentration time profile of amoxicillin and cloxacillin following single dose i.m. and i.v. administration,respectively. Cloxacillin levels were initially two-third of amoxicillin levels but were almost at par 2h post administration. Cloxacillin revealed faster absorption and distribution but slow elimination compared to amoxicillin. The volume of distribution and mean residence time (MRT) were also significantly higher for cloxacillin indicating better tissue distribution. Intramuscular route of administration proved to be superior as MRT and $Vd_{(area)}$ were almost doubled as compared to intravenous administration. The bioavailability was 73% and 63% for amoxicillin and cloxacillin by intramuscular route, respectively. Based on the pharmacokinetic data, dosage regimen with an intravenous dose of 5.0 $mg.kg^{-1}$, while an intramuscular dosage regimen with a dose of 2.5 $mg.kg^{-1}$ at every 6 h interval was recommended for Amoxicillin. An intravenous dose of 20.5 $mg.kg^{-1}$and an intramuscular dose of 15 $mg.kg^{-1}$ at every 6 h interval is recommended for Cloxacillin in goats.

Key words: Pharmacokinetics, Amoxicillin, Cloxacillin, Intravenous, Intramuscular, goats

INTRODUCTION

Amoxicillin is broad-spectrum semi-synthetic amino penicillin, which is effective against a wide range of gram-positive and gram-negative bacteria and is widely used in veterinary medicine (Pijpers *et al.,* 1989) with a low toxicity index and reliable absorption. Cloxacillin sodium is also a semi-synthetic isoxazolyl-penicillin which is penicillinase-resistant. Aminopenicillin and penicillinase resistant penicillin form a synergistic penicillin combination that is often prescribed in clinical scenario to provide an extended spectrum of efficacy, particularly against penicillin-resistant infections. Limited information is available regarding bioavailability and pharmacokinetic studies of amoxicillin and cloxacillin in various animal species but there is hardly any study describing the pharmacokinetics and bioavailability of amoxicillin and cloxacillin combination in goats which are an important component of the world livestock population.

The present study was undertaken to determine single dose pharmacokinetics and dosage regimen of Amoxicillin and Cloxacillin combination following single dose i.v. and i.m. routes of administration in goats.

MATERIALS AND METHODS

The pharmacokinetic study was conducted in six adult, non-descript, non-pregnant and non-lactating female goats (2.0-2.5 years of age and weighing 18±2.5 kg). The animals were procured from the Instructional Goat Farm of the college. The animals were reared as per IAEC standards and stall-fed with green fodder supplemented with concentrate ration and partial grazing. The animals had free access to clean fresh drinking water.

The formulation of amoxicillin and cloxacillin in the ratio of 1:1, was injected to goats i.v. @ 10 $mg.kg^{-1}$b.wt as a single bolus dose in the left jugular vein. After the intervening wash out period of one month, same animals were used for pharmacokinetic studies following i.m. administration at the same dose rate. The blood samples were collected in heparinized tubes through an i.v. cannula placed in the right jugular vein at 0, 2, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min post administration. The blood samples were centrifuged for 15 minutes at 3500 rpm for separation of plasma which was stored at -25°C till analysis. Extraction of amoxicillin and cloxacillin from plasma samples was carried out as per the method of Castro and Pedrazzoli (2003). Drug estimation in the plasma was done by HPLC.

HPLC Conditions

Amoxicillin

Mobile phase consisted of 95% phosphate buffer (0.001 mol/L, pH =4.8) and 5% acetonitrile mixture (95:5 v/v). The flow rate of mobile phase was kept at 0.8

mL.min^{-1}. Chromatography was performed at the temperature of 25°c with UV detection at 229nm Castro and Pedrazzoli (2003).

Cloxacillin

Mobile phase consisted of 70% phosphate buffer (0.001 mol/L, pH =4.7), 19% acetonitrile and 11% methanol (v/v/v).The flow rate of mobile phase was kept at 0.6 mL.min^{-1}. Chromatography was performed at the temperature of 25°c with UV detection at 225nm (Briguglio and Lau-Cam, 1984).

A standard calibration curve was obtained by plotting concentrations against the peak areas obtained for Amoxicillin and Cloxacillin. Recovery of Amoxicillin and Cloxacillin was calculated as 75% and 78% in plasma of goats, respectively.

The initial pharmacokinetic parameters were computed by least square technique as described by the methods of Baggot (1977) and Gibaldi and Perrier (1982). The pharmacokinetic analysis was done by computer software. The dosage regimen was computed by the method of Baggot (1977) and Johudein (1980). To maintain the desired therapeutic concentration Cp (min), after selecting the appropriate dosage intervals, the priming (D) and maintenance (D1) doses were calculated by using the following formulas:

$D = Cp(min).\ Vd_{(area)}.\ e^{\beta T}$; $D1 = Cp(min).Vd_{(area)}(e^{\beta T}-1)$

RESULTS AND DISCUSSION

The plasma concentration-time profile following i.v. single dose of amoxicillin and cloxacillin in goats was adequately fitted to a two compartment open model in all the animals.

The plasma concentration- time profile Amoxacillin and Cloxacillin following i.v & i.m administration in goats is shown in figure 1. Following single dose (10mg.kg^{-1}) i.v. administration, plasma concentration of amoxicillin and cloxacillin observed at 2 min post administration was 20.40μg.mL^{-1} and 14.79 μg.mL^{-1} in goats which was much lower than the concentration reported by Craigmill *et al.* (1992) following i.v. administration of amoxicillin in goats (60.63 μg.mL^{-1}). The difference in the peak concentrations of the two studies may be attributed to the different formulation used in the present study.

Table 1 shows pharmacokinetic parameters of amoxicillin and cloxacillin in plasma following its single dose (10 mg/kg) i.v. and i.m. administration in goats.The values for zero time intercept of distribution (A) and elimination (B) phase of amoxicillin (8.63 &2.34μg.mL^{-1}) and cloxacillin (12.28 & 1.74μg.mL^{-1}) following i.v. administration were lower in the present study as compared to the findings of Elsheikh *et al.* (1999) in goats(42.9μg.mL^{-1}). The short distribution half-life of amoxicillin(15.68min) and cloxacillin(8.95min) indicated a rapid distribution phase but it was slower in comparison to the observations of earlier workers (Craigmill *et al.,* 1992). The value for elimination half-life ($t_{1/2}\beta$) of amoxicillin (75.28 min) and cloxacillin (83.26min), was in accordance with earlier findings of Elsheikh *et al.* (1999). The $t_{1/2}\ \beta$ reported in the present study for cloxacillin was longer than the earlier reported values in calves (19.5 min; Daigneault *et al.,*1990). The variation may be attributed to the different physiological status of the animals due to species and climatic effect.

Table 1:
Pharmacokinetic parameters of amoxicillin and cloxacillin in plasma following its single dose (10 mg/kg) i.v. and i.m. administration in goat (n=6)

Parameters	*Units*	*Amoxicillin (Mean ± S.E.)*		*Cloxacillin (Mean ± S.E.)*	
		i.v. route	*i.m. route*	*i.v. route*	*i.m. route*
A	μg.mL^{-1}	8.63±1.91		12.28±0.90	
B/B'	μg.mL^{-1}	2.34±0.20	1.45±0.04	1.74±0.25	1.27±0.04
α/ K_a	min^{-1}	0.05±0.01	0.06±0.002	0.08±0.004	0.03±0.002
β/ K_e	min^{-1}	0.01±0.001	0.01±0.002	0.008±0.0002	0.006±0.0002
$t_{1/2}\alpha$/ $t_{1/2}k_a$	min	15.68±3.47	11.44±0.05	8.95±0.47	24.63±1.89
$t_{1/2}\beta$/ $t_{1/2}k_e$	min	75.28±11.59	121.46±2.01	83.26±2.91	104.98±2.93
AUC	μg.mL^{-1}.min	615.50±63.15	437.03±9.27	416.15±55.28	253.58±4.87
MRT	min	56.98±7.67	120.83±2.67	68.48±6.08	119.20±2.65
AUMC	μg.mL^{-1}.min^2	36621.50±7442.42	52343.40±2621.13	29004.35±5735.67	30262.60±1115.4
CL_B/(Cl/F)	mL.kg^{-1}.min^{-1}	16.90±2.12	23.01±0.52	25.36±3.34	39.48±0.77
Vd_{area}/(Vd/F)	mL.kg^{-1}	1770.33±207.80	4008.88±46.88	3016.75±336.34	5973.93±120.27
Vd_{ss}	mL.kg^{-1}	933.73±99.08	2764.95±14.89	1707.20±204.06	4701.08±47.93
C_{max}	μg.ml^{-1}		4.85±0.03		4.08±0.02
T_{max}	min		30.00		10.00
F	%		73.78±9.26		63.93±7.59.

A=Zero time intercept of distribution slope in two compartmental model; B/B' =Zero time intercept of elimination phase following i.v. and i.m. route; α/K_a = absorption/distribution rate constant following i.v. and i.m. route; β/K_e = elimination rate constant following i.v. and i.m. route; $t_{1/2}\alpha/t_{1/2}k_a$ = absorption/distribution half life following i.v. and i.m. route; $t_{1/2}\beta/t_{1/2}k_e$ =Elimination half life following i.v. and i.m. route; AUC = Total area under the time concentration curve; MRT = Mean residence time; AUMC = Area under first moment curve; Cl_B/ (Cl_B/F) = clearance/relative clearance of drug from the body following i.v. and i.m. route; $Vd_{(area)}$/ (Vd/F) = Apparent/ relative volume of distribution following i.v. and i.m. route; Vdss= Volume of distribution at steady state; C_{max}= Peak plasma concentration; T_{max}= maximum time required to attain peak plasma concentration; F= Bioavailability.

Area under the plasma concentration-time curve (AUC) for amoxicillin (615.50μg.mL^{-1} min) in goats was lower than the earlier findings (1832.73 μg.mL^{-1} min) in goats by Elsheikh *et al.* (1999). In the present study, mean residence time (MRT) for amoxicillin was 56.98 min in goats. Vd_{ss} and Vd_{area} in goats were 933.73 and 1770.33 mL.kg^{-1}, respectively, following i.v. administration. Vd_{ss} in the present study was greater than the Vd_{ss} (reported in goats (390 mL.kg^{-1} ; Elsheikh *et al.,* 1999 ; 220 mL.kg^{-1} Craigmill *et al.,* 1992).In the presnt study the clearance (CL_B) following i.v. administration in goats was 16.9 mL.kg^{-1}.min^{-1}.$CL_B$was of same magnitude(11.41 and 10.1 mL.kg^{-1}.min^{-1}) in goats has been reported by Craigmill *et al.* (1992).

The value of AUC for cloxacillin in goats following i.v. administration was 615.50 μg.mL^{-1}.min. The distribution phase was steeper while elimination was gradual for cloxacillin in comparison to amoxicillin indicating a rapid movement of the drug through the biological barriers. This hypothesis is further supported by the longer MRT and almost double Vd_{area} which reflected an excellent penetration of cloxacillin in the tissues of goats. Slow and sustained release from the tissue deposits might have played an important role in maintaining the plasma levels of cloxacillin after the initial hour post administration and thus, resulted in a steady elimination phase. The body clearance of cloxacillin was similar to that reported in calves(18.3mL.kg^{-1}.min^{-1}) by Daigneault *et al.* (1990).

Plasma concentration-time profile following i.m. administration of amoxicillin and cloxacillin in goats was adequately described by one compartment open model with first order absorption kinetics.

The peak concentrations of amoxicillin (4.88 μg.mL^{-1}) and cloxacillin (4.08 μg.mL^{-1}) in plasma after i.m. administration were observed at 30 and 10 min post administration, respectively. The peak concentrations observed were significantly low as compared to earlier reports in goat (11.03 μg.mL^{-1} at 50.94 min) by Elsheikh *et al. (*1999).

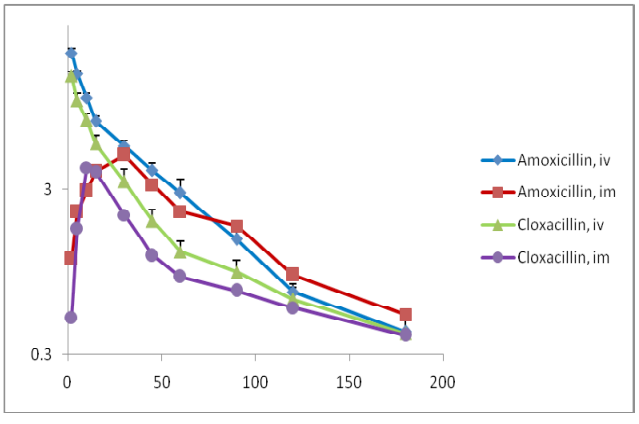

Fig 1:
Plasma conc.- time profile Amoxacillin and Cloxacillin following i.v & i.m administration in goats

The values for zero time intercept of the elimination phase (B) for amoxicillin and cloxacillin were calculated as 1.45 and 1.27 μg.mL^{-1} in goats following i.m. administration. The values of zero time intercept were much lower than the finding of Elsheikh *et al.* (1999) and Craigmill *et al.* (1992) in goats. The absorption half-lives ($t_{1/2}K_a$) in goats following i.m. administration were 11.44 and 24.63 min, respectively, indicating a rapid absorption phase. The AUC values for amoxicillin and cloxacillin were calculated as 437.03 and 253.58 μg.mL^{-1}.min, respectively. Amoxicillin was absorbed to a greater extent and at faster rate than cloxacillin. Though the peak levels showed minor differences and were attained at significantly different pace, the terminal levels of the profile revealed almost similar plasma levels for both amoxicillin and cloxacillin, suggesting immediate disposition of cloxacillin to tissue compartment following its appearance in plasma and later on, transfer of cloxacillin from peripheral to central compartment resulting in maintenance of the levels. Shorter half-life (63.23 min) and lower AUC value has been reported for amoxicillin by Elsheikh *et al.* (1999) in goats following i.m. administration.

MRT values for both amoxicillin(120.83 min) and cloxacillin(119.20min) in the present study following i.m. administration was almost double than the values obtained following intravenous administration indicating that i.m. administration resulted in longer persistence in the body. Vd_{area} for amoxicillin(4008 mL.kg^{-1}) and cloxacillin (5973 mL.kg^{-1}) following i.m. administration in goats in the present study were higher than i.v. route. Higher values for Vd_{area} explain good extravascular distribution in the body indicating the better clinical advantage. The volume of distribution in the present study were greater than the findings of Craigmill *et al.* (1992) in goats.

The intramuscular bioavailability of amoxicillin and cloxacillin in goats were 73.28 and 63.93%, respectively. The i.m. absolute bioavailability of amoxicillin in goats was quite similar to that reported in sheep (69% by Fernandez *et al.,* 2007) and horses (67% by Montesissa *et al.,* 1988). The variation in the present study may be attributed to different absorption of drug from different sites of administration. The bioavailability value for cloxacillin was comparatively low which indicates that absorption was incomplete in goats.

Based on the pharmacokinetic data, dosage regimen with an intravenous dose of 5.0 mg.kg^{-1}, while an intramuscular dosage regimen with a dose of 2.5 mg.kg^{-1} at every 6 h interval is recommended for Amoxicillin. An intravenous dose of 20.5 mg.kg^{-1}and an

intramuscular dose of 15 mg.kg^{-1} at every 6 h interval is recommended for Cloxacillin in goats.

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Research Article

HEPATOPROTECTIVE POTENTIAL OF *CYNARA SCOLYMUS* IN CISPLATIN INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present investigation was aimed to determine the alterations in antioxidant, biochemical and histopathological parameters in cisplatin (cDDP) induced hepatotoxicity and its protection by treatment with hydro-alcoholic extract of *C. scolymus*. Cisplatin (12 mg/kg BW) induced hepatotoxic rats were treated with quercetin (50 mg/kg BW), hydro-alcoholic floral extract (150 and 300 mg/kg, BW) by oral gavage. Daily oral administration of the extract for 3 days in rats significantly attenuated altered hepatic biomarkers (ALT, AST and ALP) and anti-oxidant biomarkers (TTH, MDA, CAT, SOD, GP_X, GST and GR). Pre and post treatment with plant extract at the rate of 150 and 300 mg/kg attenuated the altered levels of various enzymatic and oxidative parameters in blood and hepatic tissue but extract at higher dose levels (300 mg/kg) was more effective in restoring these parameters. These finding corroborated with reduced degenerative and necrotic changes of hepatic tissue as indicated in histopathological studies. Reduced hepatic, oxidative biomarkers and histopathological alterations indicate a good hepatoprotective potential of *C. scolymus* extract in cDDP induced hepatotoxicity in rats.

Key words: Antioxidant, Hepatoprotective, Cisplatin, *Cynara scolymus*

INTRODUCTION

Chemical induced hepatotoxicity is one of the major health problems in developed countries. Most common chemotherapeutic agents such as paracetamol, cisplatin, carbon tetrachloride, tetracyclines, thioacetamide, etc are responsible for the hepatotoxic actions. Several other conditions facilitate liver damage like viral infections (Hepatitis A, B or C), obesity, long-term alcohol use etc (Dinesh *et al.,* 2014).

Till date available modern drugs have not been able to come up with a satisfactory answer for liver disorders because of high cost and additional adverse effects. It is therefore necessary to search for alternative formulations for the treatment of liver disease to replace the currently used drugs of doubtful efficacy and safety (Dinesh *et al.,* 2014). A large proportion of medicinal compounds have been discovered with the help of ethnobotanical knowledge of their traditional uses. The rich knowledge base of countries like India and China in medicinal plants and health care has led to the keen interest by various pharmaceutical companies to use this knowledge as a resource for research and development programmes in the pursuit of discovering noble drugs (Krishnaraju *et al.,* 2005). The use of traditional medicine and medicinal plants in most of the developing countries, as a basis for the maintenance of good health has been widely observed (UNESCO, 1996). *Cynara scolymus*, belonging to family Asteraceae and commonly known by the name of 'Artichoke' has been reported to have anti-diabetic, antioxidant, anti-microbial, nephro-protective, anti-inflammatory properties etc. In addition, it provides protection against degenerative changes such as cancer. In folk medicine, *C. scolymus* has been widely used as astringent, blood cleanser, cardiotonic, detoxifier, digestive stimulant, diuretic, hypoglycemic and hypocholesterolemic as well as medicine for liver complaints (Lattanzio *et al.,* 2009). Artichoke leaf extracts have been reported to have hepato-protective, anti-carcinogenic anti-oxidative, anti-bacterial, anti-HIV, bile expelling activities as well as the ability to inhibit cholesterol biosynthesis and LDL oxidation (Martino *et al.,* 1999; Bundy *et al.,* 2009; Lattanzio *et al.,* 2009). These variable therapeutic actions of Artichoke cannot be attributed to a single component of the plant and it could be due to the presence of several bio-active components which generate synergistic pharmacological effects.

Cisplatin (cDDP) is platinum containing compound widely and efficaciously used for chemotherapy of various carcinomas, sarcomas and lymphomas (Einhorn, 2002; Pianta *et al.,* 2013). Recent studies have suggested that hepatotoxicity is also a major limiting factor in cisplatin based cancer chemotherapy (Koc *et al.,* 2005; Karasawa and Steyger, 2015). Treatment with cDDP induces the inflammatory mechanism which leads to reduction in the antioxidant levels, leading to a failure of the anti-oxidant protection against free radical damage generated by anti-cancer drugs. Further, experimental and clinical studies have demonstrated that supplementation of natural

antioxidants like curcumin (Antunes *et al.,* 2001), vitamin C (Kadikoylu *et al.,* 2004), quercetin (Francescato *et al.,* 2004; Behling *et al.,* 2006) etc protect drug induced renal damage in experimental models. Therefore, the present study was aimed to determine the hepatoprotective potential of hydro-alcoholic floral extract of *C. scolymus* in cisplatin induced hepatotoxicity in wistar rats.

MATERIAL AND METHODS

Collection and preparation of extract

The floral part of the plant *Cynara scolymus* was used. The flowers of the selected plant were collected from Pulwama region and Floriculture Park and were identified by Taxonomists, University of Kashmir. After proper identification and deposition of the voucher sample, sufficient fresh floral parts of the plant were collected in polythene bags and transported to laboratory at R.S. Pura, Jammu. In the laboratory, floral parts of *C. scolymus* were cleaned with moist cloth and were air dried in shade with temperature not exceeding 40°C for 2-3 weeks prior to extraction process. Dried parts were pre-crushed and later pulverized into fine powder using electric grinder. The dry powder was collected in polythene zip bags and stored in cool dry place. Powdered floral parts were subjected to hydro-alcoholic extraction. These powdered floral parts were weighed and were placed in thimble which was placed in the flask of soxhlet distillation apparatus and the extraction was done with 50% hydro-alcoholic solution. Extractions were done by maintaining hot plate temperature (70-80°C). The final drying was done in a rotatory evaporator. The dried extract was scrapped off and transferred to a glass container and stored in refrigerator under desiccation. The extract (0.1%) was freshly prepared in distilled water for oral administration in *in-vivo* studies in experimental animals.

Hepatotoxicity induction in experimental animals

Healthy wistar rats of either sex weighing 150-200 g obtained from Indian Institute of Integrative Medicine (CSIR lab), Jammu. The animals were provided standard pelleted ration and clean drinking water *ad libitum* and standard management conditions were provided to all the animals. The experiment was conducted on seven groups of rats with six rats in each group. Normal untreated rats (Group I) served as normal control and received only distilled water. Group II received a single intra-peritoneal dose of cisplatin (12 mg/kg BW). Group III and IV received hydro-alcoholic extract of two doses viz. 150 and 300 mg/kg BW orally. Group V and VI received plant extracts at the dose rate of 150 and 300 mg/kg BW, 1h prior and 24h and 48h after cDDP administration respectively. In Group VII, single intra-peritoneal dose of quercetin (50 mg/kg BW) was given, 6h before cDDP administration. The dose of plant extract was determined on the basis of the reported toxic dose and other pharmacological activities (Koc *et al.,* 2005; Khattab *et al.,* 2016; Najim *et al.,* 2018). The experimental protocol was approved and monitored by Institutional Animal Ethics Committee (FVSc/C-11/2456-68).

Collection and processing of samples

After 72h of cDDP administration, blood samples were collected directly from heart in a sterilized tube containing heparin. Animals were sacrificed and hepatic tissue (1g) was collected in ice cold 10ml phosphate buffer solution (0.5 M, pH 7.4). The blood samples were centrifuged at 3000 rpm for 10 minutes and plasma was collected in glass vials and stored at 4°C for the estimation of biochemical parameters and oxidative stress parameters on same day. Tissue sample was homogenized using Teflon coated homogenizer at 1000 rpm for 5-7 minutes and 10% tissue homogenate was prepared for analysis of antioxidant parameters.

Assaying of antioxidant parameters

Total thiols (TTH) level was determined in plasma and renal tissue as per the methods described by Prakash *et al.,* 2009. In brief, reaction mixture contained 900µl of EDTA (ethylene diamine tetra acetic acid) (2mM in 0.2 M Na_2HPO_4), 20µl of DTNB (5-5'- dithiobis, 2-nitrobenzoic acid) (10mM in 0.2 M Na_2HPO_4) and 100µl of fresh plasma or tissue homogenate. The reaction mixture was incubated at room temperature for 5 minutes and the absorbance was read at 412nm in UV visible spectrophotometer. A reagent blank without sample and sample blank without DTNB were prepared in the same manner. Concentration of total thiols (mM) was determined using standard (Motchink *et al.,* 1994). Similarly, malondialdehyde (MDA) levels in erythrocyte lysate or tissue homogenate were determined. The values of lipid peroxidation were expressed as nmole of MDA formed ml/h in blood and in renal tissue nmole MDA formed g in tissues/h. The other antioxidant enzymatic activities viz. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GP_X), glutathione-s-transferase (GST) and glutathione reductase (GR) were determined in blood and renal tissue using standard methods (Aebi *et al.,* 1983; Marklund *et al.,* 1974; Hafeman *et al.,* 1974; Habig *et al.,* 1974; Carlberg *et al.,* 1985). The level of reduced blood glutathione was determined as per the standard method of Beutler (Beutler, 1975).

Biochemical parameters

Biochemical parameters like alkaline phosphatase (ALP), Aspartate amino transferase (AST) and Alanine amino transferase (ALT) were determined in different groups by standard kits (Transasia Bio-Medicals Ltd, India) using Chemistry Analyzer (CHEM-

7, ERBA, Mannheim).

Histopathological studies

The histopathological studies of the renal tissues were carried out according to standard method (Drury and Wallington, 1980). Briefly, a small piece of tissue was immediately fixed in 10% formalin. These formalin fixed tissues were embedded in paraffin sectioned, stained with hematoxylin and eosin (H & E) and examined under a light microscope for histopathological assessment.

Statistical analysis

The antioxidant and biochemical parameters were depicted as mean± standard error. The results were subjected to One- way analysis of variance (ANOVA) using completely randomized design (CRD) with statistical significance at P<0.05 being tested using the Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Alterations in the plasma ALP, ALT and AST levels after giving hydro-alcoholic floral extract of *C. scolymus* in cDDP induced acute hepatotoxicity are depicted in table 1. Single intra-peritoneal cDDP administration increased (P<0.05) the levels of ALP, ALT, and AST after 72h exposure indicating hepatic impairment. Increased level of these enzymes may be due to leakage of hepatic damage caused by excessive production of ROS/RNS which may be due to high concentration of cDDP attained in liver, muscular tissue etc. Excessive ROS/RNS are also responsible for the increased lipid peroxidation of membrane leading to damage of structural and functional integrity of membranes resulting in leakage of cellular enzymes in extracellular medium (Verma *et al.,* 2013). Treatment with quercetin, a potent free radical scavenger and a metal chelator in cDDP induced hepatotoxicity decreased the alterations in ALP, AST and ALT. Administrations of floral extract at both the doses in cDDP exposed rats, lowered (P<0.05) the ALP, ALT and AST levels. At higher dose, the levels were almost restored to normal levels and these values were not significantly different from normal control group in ALT and ALP but were significantly different from normal levels in ALP.

Antioxidant system of hepatic tissue

Alterations in the levels of non-enzymatic components viz. TTH and MDA in different treatment and control groups are shown in table 2; whereas activities of CAT, SOD, GP_X, GST and GR in hepatic tissue of different groups are presented in table 3. Treatment with cDDP decreased (P<0.05) the levels of CAT where as non-significant decreased levels observed in TTH, SOD, GP_X, GST and GR but increased the levels of MDA. Administrations of *C. scolymus* floral extract raised the levels of TTH, SOD, CAT, GP_X and GR and these values non-significantly differ from control group. Higher dose (300 mg/kg) was more effective in normalizing the altered levels. MDA levels were decreased by the administration of floral extract of *C. scolymus* and high dose was more effective in normalizing the increased levels. However, significantly (P<0.05) decreased levels of higher dose of extract in MDA was observed when compared with cDDP alone treated group. Further, non-significantly increased levels of CAT, SOD, GP_X and GST were observed in treatments with extract in cDDP exposed rats. Treatment with quercetin in cDDP administered rats restored the levels of CAT, SOD, TTH, MDA, GP_X, GST and GR. The extract of *C. scolymus* is a rich source of polyphenols, flavonoids, tannins, carotenoids, etc which are having ability to scavenge free radicals like superoxide, hydroxyl and other free radicals (Lattanzio *et al.,* 2009). The increased lipid peroxidation may be resultant of excessive generation of free radicals or reduced free radicals scavenging capacity of tissues. Chiefly, the hydroxyl radical and to a lesser extent superoxide anion leads to peroxidation of membrane lipids thereby causing production of malondialdehyde (MDA) and 4-hydroxyalkenals. These substances directly induce renal tissue damage with generation of pro-inflammatory cytokines, activation of spindle cells and fibrinogenesis (Galal *et al.,* 2012) thus lead to membrane damage, protein damage, enzyme dysfunction and damage to DNA or RNA (Afroz *et al.,* 2014). In the present study, activities of SOD and CAT

Table 1:
Effect of *C. scolymus* floral extract administrations on hepatic biomarkers in the plasma of cDDP induced hepatotoxic rats

Groups	AST	ALT	ALP
Normal control	42.37^{a}±5.04	71.42^{a}±4.84	172.56^{a}±9.08
Cisplatin (12mg/kg, ip)	118.49^{d}±14.20	197.03^{b}±12.49	396.15^{c}±32.77
Extract (150mg/kg, PO)	49.50^{ab}±5.37	73.02^{a}±13.22	166.68^{a}±11.68
Extract (300mg/kg, PO)	53.55^{ab}±6.92	76.70^{a}±14.65	178.97^{a}±17.56
Extract (150mg/kg) +Cisplatin (12mg/kg)	90.38^{c}±5.64	115.46^{a}±16.75	270.64^{b}±17.92
Extract (300mg/kg) +Cisplatin (12mg/kg)	68.35^{bc}±8.25	91.35^{a}±22.33	225.77^{ab}±15.53
Quercetin (50mg/kg) + Cisplatin (12mg/kg)	59.08^{ab}±5.34	82.08^{a}±16.19	221.43^{ab}±17.69

Values are given as mean ± SE of 6 animals unless otherwise statedValues having different superscripts (a, b, c & d) in a column are statistically different from one another at 5 % level of significanceValues of AST & ALT (aspartate & alanine aminotransferase) and ALP (alkaline phosphatase) are expressed in U/L.

Table 2:
Effect of *C. scolymus* floral extract on non-enzymatic antioxidant parameters in the hepatic tissue of cDDP induced hepatotoxic rats.

Groups	Total thiols (TTH)	Malondialdehyde (MDA)
Normal control	4.92^{a}±0.58	32.53^{ab}±5.11
Cisplatin (12mg/kg, ip)	3.84^{a}±0.46	46.37^{b}±4.67
Extract (150mg/kg, PO)	5.15^{a}±0.43	31.16^{a}±5.96
Extract (300mg/kg, PO)	5.42^{a}±0.25	28.09^{a}±4.32
Extract (150mg/kg) +Cisplatin (12mg/kg)	4.30^{a}±0.57	41.69^{ab}±3.85
Extract (300mg/kg) +Cisplatin (12mg/kg)	4.94^{a}±0.20	30.80^{a}±3.43
Quercetin (50mg/kg) + Cisplatin (12mg/kg)	4.49^{a}±0.46	39.60^{ab}±4.50

Values are given as mean ± SE of 6 animals unless otherwise statedValues having different superscripts (a, b, c, d & e) in a column are statistically different from one another at 5 % level of significance. Values of TTH are expressed in Mm. Values of MDA level are expressed in nmoles MDA produced / g of tissue/ hr.

were reduced in cDDP treated rats which may be due to increased production of superoxide and peroxide radicals due to impaired mitochondrial respiratory chain reaction by cDDP. Studies also suggested that cDDP exposure decreased the activity of Cu-Zn SOD while enhancing Mn-SOD suggesting role of mitochondria in excessive production of ROS/ free radicals in cytosolic fraction (Uriu *et al.,* 2005). It is assumed that superoxide generated by cDDP is converted by SOD to H_2O_2, resulting in the reduced SOD activity. Blood GSH acts

Table 3:
Effect of *C. scolymus* floral extract on enzymatic antioxidant parameters in the hepatic tissue of cDDP induced hepatotoxic rats.

Groups	CAT	SOD	GP_x	GST	GR
Normal control	4229.86^{b}±66.20	338.19^{a}±35.80	51.04^{ab}±6.50	13.88^{a}±2.58	42.55^{a}±2.33
Cisplatin (12mg/kg, ip)	2985.45^{a}±96.65	279.76^{a}±27.10	35.99^{a}±3.54	9.45^{a}±0.86	29.59^{a}±5.28
Extract (150mg/kg, PO)	3013.44^{a}±463.81	328.51^{a}±30.86	49.32^{ab}±2.44	13.13^{a}±0.71	38.28^{a}±4.28
Extract (300mg/kg, PO)	3018.66^{a}±562.61	336.54^{a}±42.12	53.97^{b}±5.52	13.65^{a}±1.55	36.38±5.61
Extract (150mg/kg) +Cisplatin (12mg/kg)	2876.12^{a}±322.68	303.04^{a}±21.69	41.94^{ab}±4.31	10.93^{a}±1.14	29.06^{a}±3.29
Extract (300mg/kg) +Cisplatin (12mg/kg)	3042.92^{a}±348.26	327.92^{a}±40.78	48.27^{ab}±4.85	12.18^{a}±1.57	31.84^{a}±4.33
Quercetin (50mg/kg) + Cisplatin (12mg/kg)	3073.14^{a}±174.59	314.06^{a}±27.98	43.53^{ab}±5.51	10.78^{a}±0.75	36^{a}±5.92

Values are given as mean ± SE of 6 animals unless otherwise statedValues having different superscripts (a, b, c, d & e) in a column are statistically different from one another at 5 % level of significanceValues of CAT (catalase) are expressed in mol H_2O_2 decomposed /min/g tissueValues of SOD (Superoxide dismutase) and GPx (glutathione peroxidase) are expressed in Unit/g of tissueValues of GST (glutathione S transferase) are expressed in μmol of CDNB conjugate formed/ min/g of tissueValues of GR (glutathione reductase) are expressed in μmol of NADPH/min

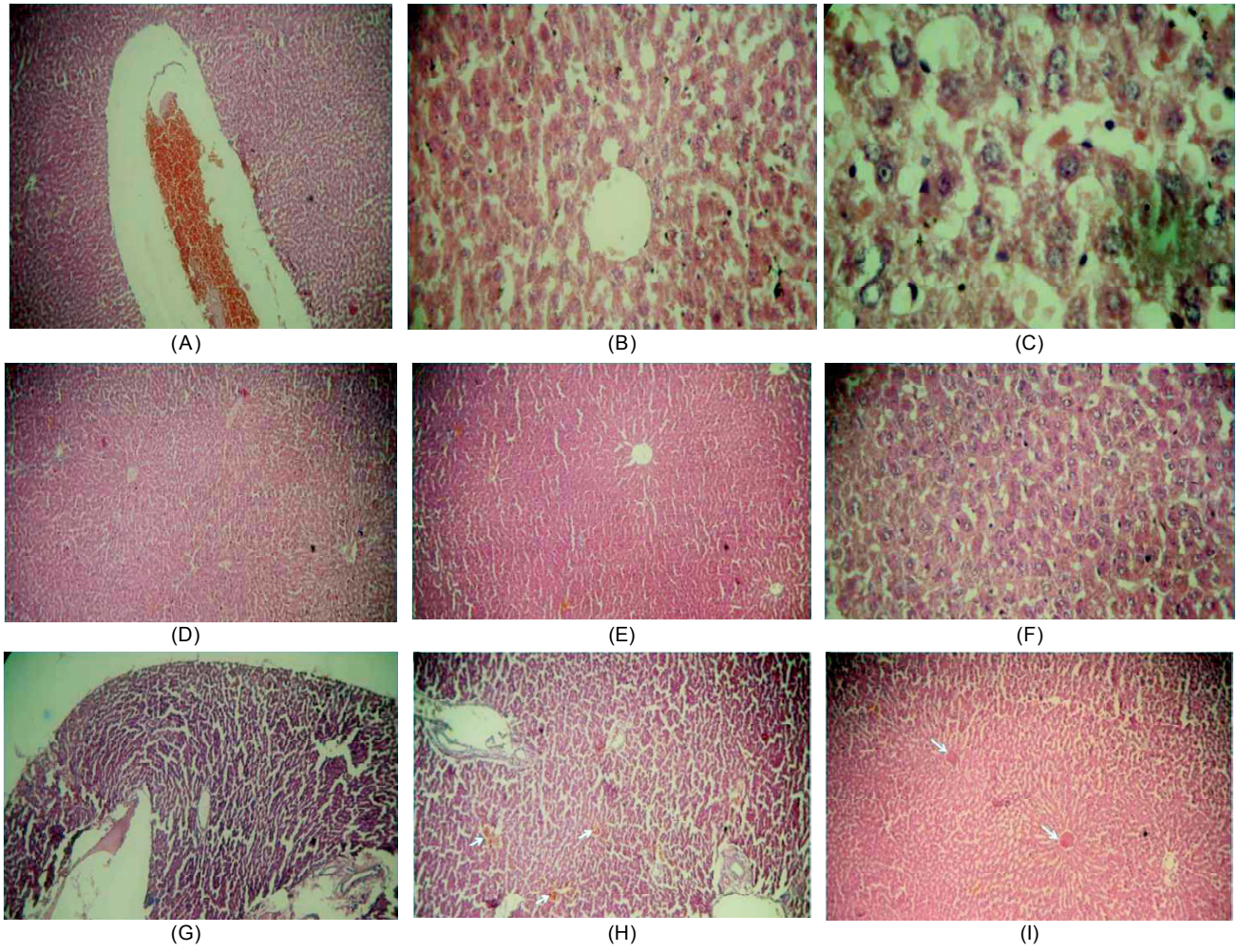

Fig 3:
Histomicrograph of H & E stained sections of the formalin fixed liver: cisplatin alone treatment (A, B, C), extract @ 150 & 300 mg/kg BW (D, E), administration of extract (150 and 300 mg/kg) along with cisplatin (F and G), quercetin (positive control) with cisplatin (H and I)

as co-factor for GST and GP_X. The reduced activities of GP_X and GST may be due to declined level of GSH, required for metabolism of free radicals.

Histopathological changes in Hepatic tissue

Liver of the normal control animals did not show any significant histopathological change. Fig. 3 depicts the histopathological changes in liver on exposure of cisplatin alone and along with plant extract at different dose levels. Intra-peritoneal administration of cDDP in wistar rats revealed marked vasodilatation and congestion of veins including widening of the sinusoidal spaces (Colak *et al.,* 2016). Mild disruption of hepatic cords with individualization of hepatocytes and degenerative changes of the hepatocytes with vacuolation and increased granularity of the cytoplasm were also observed. Similar changes were observed in hepatic tissues induced by carbon tetrachloride (Colak *et al.,* 2016; acetaminophen (Verma *et al.,* 2016); alloxan (Salem *et al.,* 2017). Pre and post treatment with hydro-alcoholic extract of *C. scolymus* in cDDP exposed rats showed the hepatocytes appear degenerated with increased cytoplasmic granularity and mild vacuolation. Similar were the findings reported with extracts of *Alstonia scholaris* in hepatotoxic rats (Verma *et al.,* 2016)

Observations of the study suggests that reduced TTH, GSH and anti-oxidant enzymes and increased MDA levels in hepatic tissue indicated reduction in antioxidant defense system on cDDP administration leading to free radicals induced acute hepatic damage as indicated in archeological alterations in hepatic tissue of wistar rats. Administrations with hydro-alcoholic floral *C. scolymus* extract minimized the cDDP induced hepatic damage as indicated by reduced MDA levels by restoring the altered hepatic antioxidant system (increased TTH, GSH, CAT, SOD, GP_X, GST and GR) during cDDP administration in animals.

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Research Article

EFFICACY OF *CARICA PAPAYA* AGAINST THIACLOPRID INDUCED HISTOARCHITECTURAL CHANGES IN BRAIN AND SPLEEN OF RATS

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ABSTRACT

The study was carried out to investigate efficacy of *Carica papaya* against thiacloprid induced histoarchitectural changes in brain and spleen of rats. Wistar rats were divided in three groups having six rats in each group. Group I served as control (corn oil administered as vehicle). Group II served as thiacloprid treated group (@ 22.5 mg/kg b.wt. orally) and group III (experimental group) thiacloprid along with papaya leaves extract (@ 252mg/kg b.wt.orally) were administered. The thiacloprid treated groups showed marked neuronal degeneration and congestion of blood vessels in the neuropil of cerebral cortex and exhibited moderate to mild lymphocytic depletion in periarteriolar lymphoid sheaths (PALS) of white pulp in the spleen. Papaya treatment showed improvement in histopathological changes of brain and spleen. The ethanolic extract of *Carica papaya* has the potential to restore the histoarchitectural changes in brain and spleen caused by sub-acute thiacloprid toxicity.

Key words: Thiacloprid, Papaya, sub acute toxicity, rats

INTRODUCTION

The neonicotinoids are a new major class of highly potent insecticides that are used for crop protection against piercing-sucking insects of cereals, vegetables, tea and cotton, and for flea control in cats and dogs. Currently the best known neonicotinoid is imidacloprid, which is an active ingredient in commercial preparations such as Confidor, Gaucho, Prestige, Admire, and Premier which are increasingly used in agriculture (Tasei *et al.,* 2000). It was known as neonicotinoids, based on its similarity to nicotine in terms of structure and action (Rose, 2012).

Thiacloprid, a new neonicotinoid insecticide belonging to the group of active ingredients, the cyanoamidines is neurotoxic (Tomizawa and Casida, 2005) and is effective on contact and also via stomach action. It produces its action by binding agonistically to the nAChRs in the CNS of insects (Zhang *et al.*, 2000). Thiacloprid is presently the most important commercial product because of its high efficacy against insects. It has outstanding potency and systemic action for crop protection against piercing-sucking pests and is also highly effective for flea control on cats and dogs (Tomizawa and Casida, 2005). In 2007, thiacloprid was registered in more than 50 countries for use in agriculture, horticulture etc.

In current scenario, herbal drugs being cheap and locally available are playing an important role in health care programmes. Recently, WHO (World Health Organization) estimated that 80 percent of people worldwide rely on herbal medicines for some aspect of their primary health care needs. Treatment with medicinal plants is considered safe as there is no or minimal side effects. These remedies are in sync with nature, which is the biggest advantage. The golden fact is that, use of herbal treatments is independent of any age groups.

Papaya (*Carica papaya* L.) is a popular and important fruit tree in tropical and subtropical parts of the world. The fruit is consumed worldwide as fresh fruit and vegetable or used as processed product. The fruit is healthy and delicious and the whole plant parts including fruit, root, bark, peel, seeds and pulp are also known to have medicinal properties. Most of the benefits of papaya are owed due to high content of vitamin A, B and C, proteolytic enzymes like papain and chymopapain which have antiviral, antifungal and antibacterial properties. During the last few years, major insight has been achieved regarding the biological activity and medicinal application of papaya and now it is considered as a valuable neutraceutical fruit plant (Vij and Parashar, 2015).

Recent studies suggest that papaya provides protective effect on neurons by suppression of ERK signaling mechanism (Zhao *et al.,* 2016), modulate non–functional and functional immune response (Jayasinghe, *et al.,* 2017), nephroprotective effect (Naggayi *et al.,* 2015) and decrease ischaemia / reperfusion – induced injury (Allahyari *et al., 20*14). Papaya leaves also increase platelet count in dengue fever and prevents thrombocytopenia (Gadhwal *et al.,* 2016).

Since efficacy of papaya against toxicity induced by thiacloprid is scarcely available in literature, therefore present work was conducted with the objective

Table 1:
Experimental design for evaluating efficacy of extract of papaya leaves against sub-acute toxicity of thiacloprid

Groups	Treatment	No.of rats	Dose(mg/kg b.wt.)Oral route	Feeding schedule
I	Control (corn oil)	6	10ml/kg	0 - 28 days
II	Thiacloprid	6	22.5	0 - 28 days
III	Thiacloprid + extract of Papaya leaves	6	22.5+252	0 - 28 days

to evaluate ameliorating potential of papaya against histopathological alteration induced by sub-acute exposure of thiacloprid in brain and spleen of rats.

MATERIALS AND METHODS

All the experimental procedure, housing and management of the rats were strictly carried out according to the recommendation and approval of the institutional animal ethical committee (IAEC).

Adult wistar rats weighing 120-140 g were used for the study. Rats were housed in polyacrylic cages in a group of 6 rats per cage in the Department of Veterinary Pharmacology & Toxicology. Bedding material (wheat straw) was changed on alternate day. The animals were provided with feed and water *ad libitum* and maintained at room temperature with a natural light-dark cycle. Rats were acclimatized to laboratory conditions for 7 days before the experiment was conducted.

Herbal Extract

(i) Collection of plant materials: Papaya leaves were procured from nursery**.**

(ii) Extraction:

The dried leaves of papaya and seeds of fenugreek were powdered and extracts were prepared by rotary vacuum evaporator. The percentage yield of ethanolic extract of papaya leaves were found to be greater than aqueous extract so the said extract were used in the experiment.

Toxicological Agents

Thiacloprid

Technical grade Thiacloprid (96.8% pure) was used in the experiment and desired concentration was prepared in corn oil.

Experimental design

Eighteen rats were randomly divided into 3 groups (6 rats/group) as shown in Table 1. Group I served as control in which corn oil (acting as vehicle of thiacloprid) was administered. Group II served as thiacloprid treated group (@ 22.5 mg/kg b.wt.orally). Similar dose of thiacloprid was used by Aydin (2011) in rats to induce toxicity. In case of group III (experimental group) thiacloprid along with papaya leaves extract (@ 252mg/kg b.wt.orally) were administered.

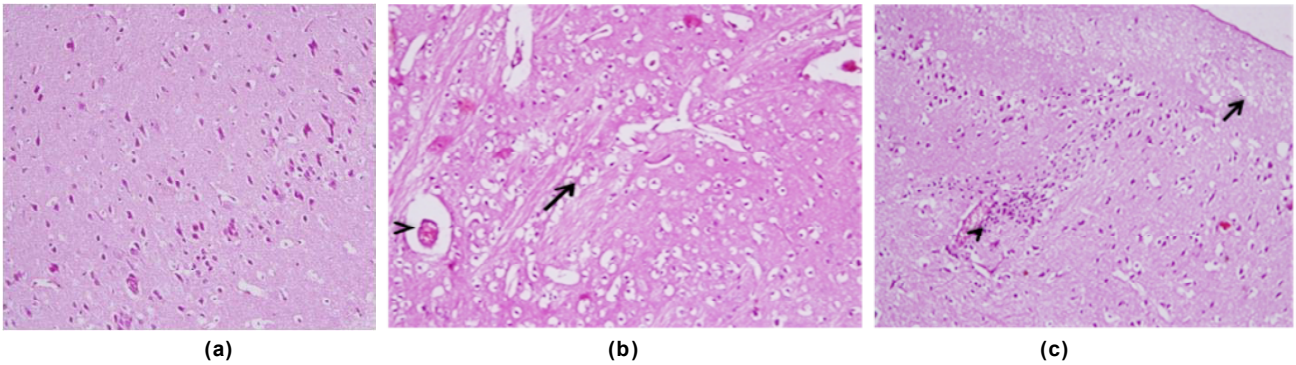
Histopathology

After blood collection, rats were sacrificed by cervical dislocation. Spleen and brain were collected and preserved in 10% formalin. Paraffin embedded section of these tissues 5-6 µm thickness were cut and stained with haematoxylin and eosin.

RESULT AND DISCUSSION

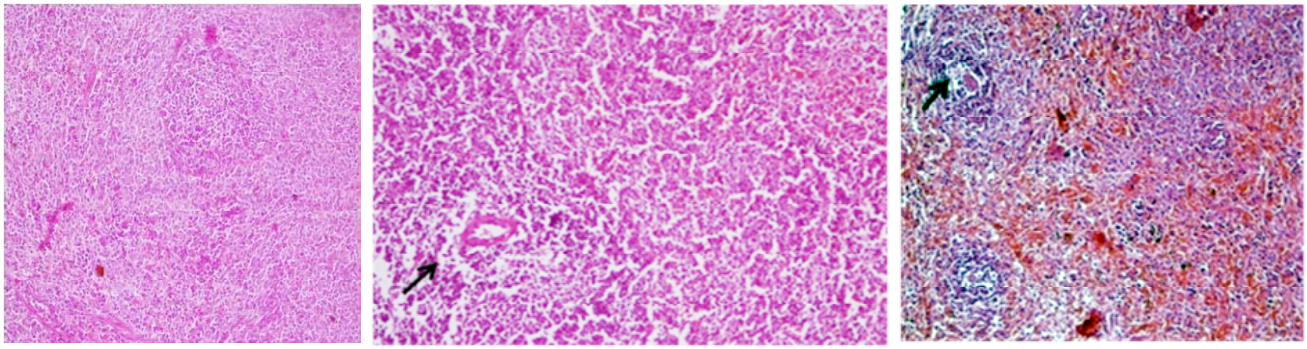
Brain: Histopathological lesions in the brain of control and other treatment groups are presented in plate 1. The control group displayed intact neuron and glial cells in the neuropil of cerebral cortex whereas in thiacloprid treated groups there were marked neuronal degeneration and congestion of blood vessels in the neuropil of cerebral cortex. Papaya treatment showed improvement in histopathological changes of brain by diminishing the neuronal degeneration in the neuropil of cerebral cortex.

Subacute toxicity of thiacloprid resulted in neuronal degeneration and congestion of blood vessels



(a) (b) (c)

Plate 1:
Histopathological sections of brain (28 days study:(a) intact neurons and glial cells in the neuropil of cerebral cortex in control group; (b) marked neuronal degeneration (arrow) and congestion of blood vessels (arrow head) in the neuropil of cerebral cortex in Thiacloprid group; (c) moderate neuronal degeneration (arrow) and congestion of blood vessels (arrow head) in the neuropil of cerebral cortex in Thiacloprid+Papaya group (H & E x 200)



(a) (b) (c)

Plate 2:
: Histopathological sections of spleen (28 days study:(a) normal red pulp and adequate lymphocytes in PALS of white pulp in the spleen in control groups respectively; (b) showing moderate depletion of lymphocytes (arrow) in PALS of white pulp in the spleen in Thiacloprid group; (c) mild depletion of lymphocyte in germinal centre (arrow) of PALS of white pulp in the spleen in thiacloprid +papaya group (H & E x 200)

in the neuropil of cerebral cortex. Similar findings were reported by Goyal *et al. (*2010) where cerebral hemisphere revealed changes comprising of mild neuronal degeneration with surrounding glial cells in *Gallus Domesticus* due to thiacloprid toxicity. Similar findings were reported by Soujanya *et al.* (2012) where imidacloprid treatment resulted in histological and ultrastructural alterations in brain of male rats.

Papaya co-treatment restored the normal histopathological structure of brain. It could be due to activation of Nrf2 in astrocytes by papaya which plays an important role in brain homeostasis (Murakami *et al.,* 2016). These results demonstrated papaya having neuro-protective action.

Spleen

Histopathological lesions in the spleen of control and treatment groups are presented in plate 2. The control group showed normal red pulp and adequate lymphocytes in periarteriolar lymphoid sheaths (PALS) of white pulp in the spleen whereas in thiacloprid treated groups exhibited moderate to mild lymphocytic depletion in PALS of white pulp in the spleen. Papaya co-treatment in thiacloprid treatment groups displayed improvement in histopathological changes of spleen by diminishing the lymphocytic depletion in PALS of white pulp.

Subacute toxicity of thiacloprid resulted in moderate to mild lymphocytic depletion in PALS of white pulp in the spleen. The findings were in agreement with Mohany *et al.* (2011) who reported that histopathological changes of the spleen tissues of the imidacloprid-treated rats displayed reduced numbers of lymphocytes, some of which appeared to be pyknotic.

Papaya co-treatment in thiacloprid treated groups restored the normal histopathological structure of spleen. It may be attributed to the protective effect of papaya on spleen (Oduola, *et al.,* 2010, Choudhary *et al.,* 2001).

Thus, it can be concluded from the study that ethanolic extract of *Carica papaya* has the potential to restore the histoarchitectural changes in brain and spleen caused by sub-acute thiacloprid toxicity

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Research Article

SAFETY EVALUATION OF OLEIC ACID IN ISOPRENALINE INDUCED MYOCARDIAL INJURED RATS

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ABSTRACT

The lifestyle diseases have emerged as a naive threat to the human beings and to curtail the incidence and emergence of these disorders such as cardiovascular disorders and other diseases; the use of several agents has been advocated. Oleic acid has role as anti-inflammatory and antioxidant. Therefore, present study was conducted to ascertain the safety haematological and analytic profile of oleic acid treatment in myocardial infarction cases in rats. The study showed no significant alteration in hematogram and also revealed the presence of oleic acid in cardiac tissue as retention time by GC-MS found to be around 41.9 min. Thus, study confers the utility of oleic acid in the cases of myocardial infarction and other cardiovascular diseases.

Key words: Isoprenaline, myocardial infarction, Oleic acid, GC-MS.

INTRODUCTION

The cardiovascular diseases are the most common cause of death throughout the world (Reeve and Rafferty, 2005). According to the World Health Organization (WHO), 31% global deaths were reported due to the cardiovascular diseases in 2012 and out of these 80% cases are linked with the ischemic heart disease. The mechanistic study has revealed the role of oxidative damage and free radical induced injury along with the inflammatory reactions in the cascade of development of cardiovascular diseases. Olive oil has anti-inflammatory and antioxidant properties, thus have ameliorative effect over cardiovascular system. Oleic acid is an important component of olive oil; thus has immense potential to counter the ill effects of myocardial infarction and other disorders. Earlier we have reported the cardio-protective role of oleic acid in myocardial injury and its underlying mechanism (Singh *et al.,* 2019). Thus the present study was conducted in conjunction with the major study to evaluate the toxic studies with reference to haematological parameters and distribution of oleic acid to the cardiac site of action.

MATERIALS AND METHODS

The study was conducted in 120 adult male Wister rats (170-200 grams), procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana; were randomly and equally divided into six groups with twenty animals in each group and subjected to different treatments in different treatment protocol in different groups as mentioned below:

Induction of myocardial infarction and sampling of blood

The myocardial infarction were induced in the animals of Gr-II, V and VI with isoprenaline (@ 110 mg/kg b.wt.) for two consecutive days (20thand 21st day) via intra-peritoneal route at 24 h interval. Animals were sacrificed after 24 h of last dose of isoprenaline administration. Blood samples were collected from inner canthus of eye of rats of respective groups on 22nd day of experiment under light ether anesthesia to assess haematological parameters. Heart samples from different groups were collected under surgical stage of anesthesia.

Detection of oleic acid in heart homogenates

Heart samples from different groups were subjected to gas-chromatography mass spectrophotometery (GC-MS) analysis to detect oleic acid and its metabolites present, if any.

Sample preparation

Heart samples from different groups were processed for GC-MS analysis by fatty acid methyl esters (FAME) synthesis method. Briefly, 500 mg fresh heart tissue sample was taken in a screw capped tube and 0.35 ml of 10 N KOH and 2.65 ml methanol was added and mixed well. Tubes were then incubated at 55°C for 1.5 h in water bath with vigorous shaking for every 20 min to properly permeate, hydrolyze and dissolve the sample. Samples were then kept in cold tap water for cooling and 0.29 ml of 24 N H_2SO_4 was added and mixed well. The tubes were then incubated at 55°C for 1.5 h in water bath with vigorous shaking for 5 sec. in every 20 min to properly permeate, hydrolyze

Table 1:
Experimental design of the study

Group	Description	Treatment and route of administration
I	Healthy Control (HC)	Distilled water (DW) orally for 21 days
II	Isoprenaline (ISO)	Isoprenaline @ 110 mg/kg *i.p.* on 20th and 21st day at 24 h interval
III	HC + Oleic acid -5 (OA-5)	Oleic acid @ 5 mg/kg, orally for 21 days
IV	HC + Oleic acid-10 (OA-10)	Oleic acid @ 10 mg/kg, orally for 21 days
V	Oleic acid -5 (OA-5) + ISO	Oleic acid @ 5 mg/kg, orally for 21 days + isoprenaline @ 110 mg/kg *i.p.* on 20th and 21st day at 24 h interval
VI	Oleic acid-10 (OA-10) + ISO	Oleic acid @ 10 mg/kg, orally for 21 days + isoprenaline @ 110 mg/kg *i.p.* on 20thand 21st day at 24 h interval

and dissolve the sample. Three millilitre of hexane was then added after cooling the samples and then mixed properly by using vortex mixer for 5 min. After centrifugation for 5 min hexane layer was taken in GC vial and vials were capped and placed at -20°C until further analysis.

Sample analysis

The processed samples (1µl) were analysed using GC-MS (Agilent system 5975 VL MSD and 789O A GC system) equipped with 5 % phenyl methyl siloxan capillary column (325 °C; 30 m x 320 µm x 0.25 µm). Hydrogen was used as carrier gas for GC-MS analysis. The conditions used for GC-MS analysis were as follows:

For GC

Temperature limits	:	60 to 325 ˚C
Injector temperature	:	250 ºC (splitless mode)
Carrier gas	:	Hydrogen
Flow rate	:	2.5 ml/min
Injection volume	:	2 µl
Oven temperature	:	100 ˚C for 3 min with an increase of 10 ˚C/ min to 250 ˚C for 3 min then Increase @ 30 ˚C/ min to 290 ˚C with 10 min hold
Run time	:	60 min

For MS

Electron ionization energy	:	69.92 ev
Mass scan (m/z) fragements	:	45 to 650 Da
Solvent delay	:	2.5 min
MS source temperature	:	230 ˚C (max -250 ˚C)
MS quad temperature	:	150 ˚C (max -200 ˚C)
Run time	:	60 min

The identification of compounds was done by using NIST -08 library matches with reference to the mass charge (m/z) ratio of the components obtained in GC-MS analysis.

RESULTS

The present study revealed that myocardial injury following administration of isoprenaline (@ 110 mg/kg. wt.; *i.p*) significantly (p<0.05) decreased haemoglobin (Hb), total erythrocyte count (TEC) and lymphocyte percentage. However, total leukocyte count (TLC) and granulocyte percentage were significantly (p<0.05) increased in ISO group as compared to control animals (Table 2). Oleic acid pre-treatment either at lower (5 mg /kg. b.wt.) or higher (10 mg/kg b.wt.) did not produce any significant alterations in Hb, TEC, TLC, lymphocyte and granulocyte count as compared to isoprenaline exposed group (Gr II). Further, monocytes percentage was not altered significantly in any of the treatment groups as compared to control animals (Table 2). The present findings narrated that exposure to oleic acid for 21 days alone either at lower or higher dose did not have any significant effect on haematological profile as compared to control animals.

The GC-MS analysis using flame ionized detector (FID) revealed the retention time of oleic acid in hexane was found to be 41.9 min in all the groups. The chromatogram and corresponding reports of GC analysis of heart homogenates from different groups are illustrated in Fig. 1, 2 and 3.

DISCUSSION

The exposure of oleic acid for a period of 21 days did not produce any significant alterations in haematological values suggested that the exposure of oleic acid for around three weeks daily would not produce

Table 2:
Effect of oleic acid pre-exposure on haematological parameters in myocardial injury in rats.

Group	(n=6)	Hb (g/dl)	TEC (10^6/µl)	TLC (10^3/µl)	Lymphocyte (%)	Monocyte(%)	Granulocyte (%)
I	Control	13.5^{a} ± 0.17	8.92^{a} ± 0.16	12.48^{a} ± 0.17	63.05^{a} ± 0.98	4.38^{ab} ± 0.77	32.56^{a} ± 1.00
II	ISO	11.82^{b} ± 0.22	7.26^{b} ± 0.33	17.73^{b} ± 0.25	54.46^{b} ± 0.35	3.96^{a} ± 0.08	41.58^{b} ± 0.67
III	OA-5	13.11^{a} ± 0.23	8.72^{a} ± 0.14	11.94^{a} ± 0.29	61.36^{a} ± 0.85	4.23^{ab}± 0.33	35.75^{a} ± 0.61
IV	OA-10	13.34^{a} ± 0.25	8.70^{a} ± 0.11	12.08^{a} ± 0.22	59.76^{a} ± 1.98	5.10^{b} ± 0.23	35.76^{a} ± 0.96
V	OA-5 + ISO	11.50^{b} ± 0.25	7.08^{b} ± 0.21	15.98^{c} ± 0.39	53.582^{b} ±0.82	4.50^{ab}± 0.44	41.18^{b} ± 0.71
VI	OA-10 + ISO	11.43^{b} ± 0.24	6.81^{b} ± 0.17	16.45^{c} ± 0.38	47.83^{b} ± 2.00	4.33^{ab} ±0.19	43.21^{b} ± 1.6

Data are expressed as mean ± SEM, n = 6. Data were analyzed by one way analysis of variance (ANOVA) followed by Tukeys post-hoc test. Mean values with different superscripts within same column differed significantly (p<0.05).

deleterious effect over hematogram. Oleic acid (OA), a mono unsaturated fatty acid (MUFA) of omega-9 family, is the primary component of olive oil which is widely used in Mediterranean diet (Waterman and Lockwood, 2007). The Mediterranean diet is reported to reduce the cardiovascular diseases by dampening the circulating inflammatory biomarkers and oxidative stress (Nakbi *et al.,* 2010; Urpi-Sarda *et al.,* 2012). Thus maintaining the hematological values at normal range is a suggestive of beneficial and protective role of oleic acid in myocardial infarction cases. The GC-MS analysis revealed that the administered dose of oleic acid is sufficient to reach to its site of action in order to produce prophylactic response

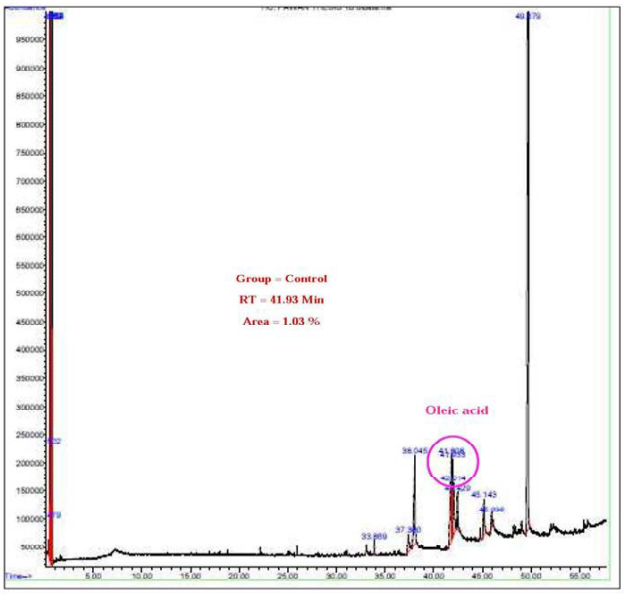

Fig. 1:
Chromatogram showing percentage peak area of oleic acid as detected following GC analysis of heart homogenates from control group.

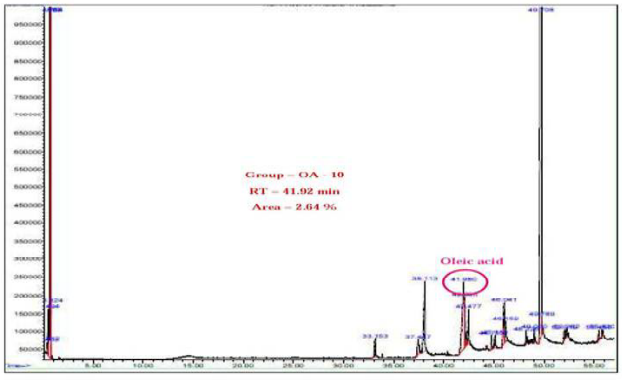

Fig. 2:
Chromatogram showing percentage peak area of oleic acid as detected following GC analysis of heart homogenates from OA-10 group (Gr IV).

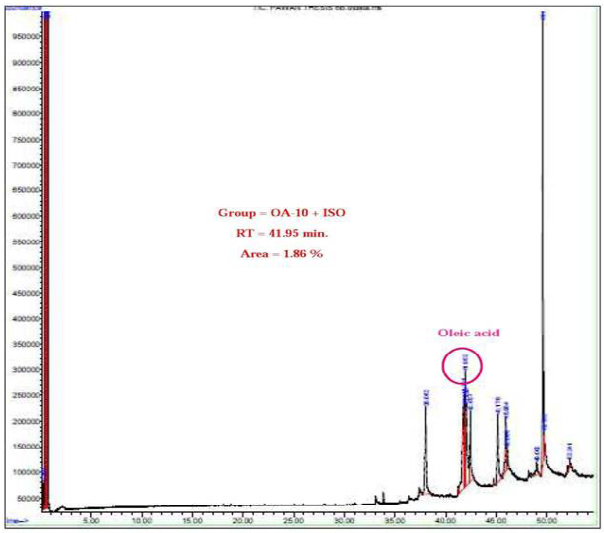

Fig. 3:
Chromatogram showing percentage peak area of oleic acid as detected following GC analysis of heart homogenates from OA-

following myocardial exposure in rats. Further studies are warranted to ascertain the role of different metabolites in the molecular mechanistic pathways as interplaying domains.

It is concluded from the above study that the oleic acid has the protective role in cardiovascular disorder as suggested by the no significant alterations and also by the analysis of the oleic acid in the cardiac tissue that is the major target organ of the study.

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Research Article

OOCYTE RECOVERY AND THEIR QUAILITY IN RELATION TO INFLUENCE OF CORPUS LUTEUM

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ABSTRACT

Experiment was conducted to assess the effect of presence of corpus luteum on ovarian follicular population, oocyte recovery and their quality in buffaloes. A total of 300 ovaries were collected, 165 were bearing corpus luteum (CL) and 135 without CL. A significantly ($P<0.05$) more number of oocytes were recovered (72.96%) from the ovaries without corpus luteum compared to ovaries bearing CL (67.42%). Usable oocytes (grade A and grade B) could also be recovered from ovaries without corpus luteum (531) as compared to ovaries bearing CL (363).Recovery of usable oocytes per ovary was 3.22±0.1 and 2.69±0.08 from the ovaries without CL and with CL, respectively. Present experiment concluded that presence and absence of corpus luteum (CL) on the ovaries has significant impact on follicular development as well as quality of oocyte recovered, thus this could be use to predetermine the recovery and qualities of oocytes in *in vitro* studies using such materials.

Key words: Buffalo, corpus luteum, oocyte, follicle

INTRODUCTION

Large numbers of good quality oocytes suitable for invitro studies are needed for successful production of buffalo pre-implantation embryos. In the cow and goat ovaries containing a CL, have yielded lower numbers of oocytes than ovaries without CL but such information is scanty in buffaloes (Pierson and Ginther, 1987). There are several reports indicating that presence or absence of CL on ovary influence the growht and number follicles (Souza *et al.,* 1996; Raza *et al.,* 2001) The present study reports the possible impact of CL on recovery of oocytes per ovary and their quality, collected from abattoir.

MATERIALS AND METHODS

Buffalo ovaries were collected from local abattoir and transported to laboratory in thermos flask at about 35^0C. A total of 300 ovaries were used for this experiment. Out of these ovaries, 165 were without CL while 135 were bearing CL.

The diameter of visible surface follicles was measured, for both type of ovaries, with vernier calipers and the surface follicles were classified into 3 categories i.e. small (1-4 mm), medium (>4-8 mm) and large (>8 mm) according to Abdoon and Kandil (2001). The follicles were aspirated and pooled separately as ovaries were categories in to two; with or without corpus luteum. Oocytes collected were graded into different categories (Gupta *et al.,* 2002) under stereo-zoom microscope as follows:

Grade A: Oocytes with 4–5 layers of cumulus cells with homogenous and evenly granular grey ooplasm;
Grade B: Oocytes not having much compaction, with 2–3 layers of cumulus cells surrounding the zona pellucida and having evenly granular ooplasm;
Grade C: Oocytes with 1–2 layers of cumulus cells or partially denuded with irregular dark ooplasm;
Grade D: Oocytes without cumulus cells or with highly expanded or scattered cumulus cells and having irregular dark ooplasm.

Recovery and quality of oocytes obtained in different groups were recorded and compared.

RESULTS AND DISCUSSION

Detailed results of present study are given in table 1. It was found that number of follicles differed insignificantly between ovaries with or without CL. The values being 8.89±1.02 and 8.5±1.21 per ovary, when CL was present and absent respectively. These results are in accordance with Dominguez (1995), who reported that presence of a CL did not affect follicle number between or within cows. In contrast, several researchers have reported that the CL bearing ovary in cows contains more follicles (Pierson and Ginther, 1987; Savio *et al.,* 1981). Others also reported that ovaries bearing CL contains less number of follicles than the ovaries, which were not bearing CL (Moreno *et al.,* 1993; Amer *et al.,*2008; Mekwana *et al.,*2012). This difference may be attributed to the fact that although luteal structures were identified during ovarian examination, their functional status was not confirmed, thus, cows in pro estrus contained a regressing CL that may have been defined as a CL if it still showed a relatively large size on the ovarian surface.

In the present study, significantly greater number of oocytes were recovered (72.96%) when corpus luteum

Table 1:
Effect of presence of corpus luteum on follicular population and oocyte recovery.

S.No.	Attributes		Corpus Luteum absent	Corpus Luteum present
1.	No. of Ovaries		165	135
2.	No. of follicles	small	1155	1036
		medium	223	134
		large	24	18
		TOTAL	1402	1188
3.	No. of follicles per ovaryMean ± SE	8.56±1.21[a]	8.89±1.02[a]	
4.	Oocytes recovered	GRADE A*(N)	24.34%[a](249)	18.26%[b](146)
		GRADE B*(N)	27.57%[A](282)	27.10%[A](217)
		GRADE C*(N)	25.7%[B](263)	31.27%[A](254)
		GRADE D*(N)	22.39%[A](229)	22.97%[A](184)
		TOTAL	1023	801
		OOCYTE RECOVERY (%)	72.96[A]	67.42[B]
5.	TOTAL USABLE OOCYTES (GRADE A + grade B) recovered		531	363
6.	Usable oocytes (grade A + grade B) recovered/ ovary Mean ± SE		3.22±0.1[a]	2.69±0.08[b]

The values bearing different superscripts in the row differ significantly (P<0.05)
*Per cent values calculated out of recovered oocytes.

was absent as compared to that, when corpus luteum was present on the ovary (67.42%). Similar observations have been made in cattle (Moreno *et al.,* 1993) and in buffaloes (Das *et al.,* 1996; Singh *et al.,*2001; Nandi *et al.,* 2002). This is because the follicular development is restricted as, lutein cells occupy the most of the portion of the ovary (Kumar *et al.,* 1997). In contrast, Boediono et a1. (1995; Mekwana *et al.,*2012) found no difference between the mean number of the oocytes per ovary between CL bearing and non CL bearing ovaries after aspiration. The present study indicated significantly higher oocyte recovery rate in ovaries, where corpus luteum was absent.

Recovery of usable oocytes per ovary was also significantly higher in ovaries not bearing CL (3.22±0.1) than ovaries bearing CL (2.69±0.08). Similar observations have been made by Agarwal (1992), Moreno *et al.* (1993) and Das *et al.* (1996). This could be explained on the basis of the fact that, the CL may act on the follicles to alter their growth rate to result in atresia (Rexroad and Casida, 1975) and hence poor number and quality of oocytes.

On the basis of our results, it may be concluded that the recovery of per cent total oocyte, total usable oocytes (grade A + grade B) and usable oocytes per ovary were significantly higher in absence of corpus luteum. However, number of follicles per ovary was not affected by corpus luteum.

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