Research Article

DAIDZEIN PREVENTS SEPSIS-INDUCED INSULIN RESISTANCE IN MICE

MOHAMMAD IRFAN SHAH[1], RUSHEEBA MANZOOR[2], HARI KUMAR S[1], SUBHASHREE PARIDA[1], THAKUR UTTAM SINGH[1], SANTOSH KUMAR MISHRA[1]

[1]Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, U.P, India
[2]Division of Veterinary Microbiology and Immunology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Kashmir Shuhama Campus, Srinagar- 190 006, India
Corresponding Author: Email: smishraivri@rediffmail.com

ABSTRACT

Present study was carried to investigate the effect of daidzein on sepsis-induced insulin resistance in mice. Sepsis was induced in Swiss albino mice by caecal ligation and puncture (CLP) method and the time of peak hyperglycemia in sepsis was observed. Animals were divided in to three groups. Group I was kept as sham control, group II (CLP) was administered vehicle (50% DMSO@3ml/kg by I/P injection) 2 hr before surgery and group III (daidzein+CLP) was treated with daidzein (@ 3mg/kg b.w. by I/P injection) 2hr before surgery. Effect of pre-treatment with daidzein in septic mice was studied by measuring serum glucose, insulin level and liver glycogen. In septic animals hyperglycemia was observed at 2 h post surgery and daidzein pre-treatment resulted in partial restoration of glucose level. However, daidzein was unable to restore the glycogen level in liver but completely restored the insulin level in blood. Sepsis caused significant ($p<0.05$) reduction in the insulin receptor mRNA expression in comparison to the Sham. Daidzein pre-treatment showed significant ($p<0.05$) increase in insulin receptor mRNA expression. The results indicate that the phytoestrogen daidzein has the potential to improve insulin resistance in mouse model of sepsis

Keywords: CLP, Sepsis, Daidzein, Hyperglycemia

INTRODUCTION

Sepsis is one of the most prevalent diseases and one of the main causes of death among critically ill patients (Mayr *et al.*, 2013). Sepsis represents a continuum beginning with a host–pathogen interaction that triggers a complex interplay between pro-inflammatory, anti-inflammatory, and apoptotic mediators (Gariani *et al.*, 2013). It is a common condition in neonatal animals, horses with colic, and cows with displacement of abomasum and abomasal volvulus. Sepsis is a clinical syndrome that complicates severe infection and is characterized by systemic inflammation and widespread tissue injury. Severe sepsis is manifested by organ dysfunction (i.e. hypoperfusion, tissue hypoxia, lung injury, etc.), while septic shock is a type of severe sepsis marked by hypotension despite fluid resuscitation (Russel, 2006). In Indian context, hospital mortality and 28-day mortality of severe sepsis were found to be 65.2% and 64.6%, respectively within the period of June 2006 to June 2009 (Todi *et al.*, 2010). Treating patients with severe sepsis and septic shock has been a great challenge to intensive care specialists. Management of sepsis relies on the early identification and treatment of the underlying causative infection, adequate and rapid hemodynamic resuscitation, support of associated organ failure and modulation of the immune response with drotrecogin alfa (activated), and corticosteroids in severe septic shock (Vincent *et al.*, 2011). Despite intense research in to the pathogenesis of sepsis, the current therapy for this devastating syndrome is primarily supportive and mortality remains high (Goldenberg *et al.*, 2011).

In sepsis, profound alterations in the metabolic pathways occur leading to hyper metabolism, enhanced energy expenditure and insulin resistance. Stress induced hyperglycemia and insulin resistance are almost universal findings in patients with sepsis (Leonidou *et al.*, 2008) and occur by the action of circulating cytokines and counter-regulatory hormones released under stress conditions (Preiser *et al.*, 2010). The clinical hallmarks are hyperglycemia, hyperinsulinemia, hyperlactatemia and enhanced protein catabolism (Lind and Lithell, 1994) and the hyperglycaemic condition may lead to inflammatory stress and aggravate the sepsis (Kyle *et al.*, 2010).

Daidzein is a phytoestrogen belonging to the class isoflavone. It binds to estrogen receptors and has both weak estrogenic and weak anti-estrogenic effects. Exposure to daidzein occurs principally through foods made with soybeans and soy protein. Daidzein plays a beneficial role in diabetes (Cheong *et al.*, 2014). Unsweetened soy foods and isoflavones have been shown to have a protective role on the risk of type 2 diabetes (Mueller *et al.*, 2011). In the present study, we examined the effect of daidzein on sepsis-induced insulin resistance.

MATERIALS AND METHODS

Animals: Healthy adult male Swiss albino mice weighing 25-30 g (procured from Laboratory Animal Resource Section, IVRI, U.P.) were employed in the present study.

They were housed in polypropylene cages with free access to standard feed and water. The mice were acclimatized to the laboratory conditions seven days prior to study.The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC).

Experimental protocol: All the animals were randomly divided in to three groups. Group I, Sham control, was administered normal saline (@3ml/kg body weight by I/P injection) 2 h before surgery. Group II (CLP) mice were administered vehicle (50% DMSO@3ml/kg by I/P injection) 2 h before surgery. Group III (daidzein+CLP) was treated with daidzein (@3ml/kg by I/P injection) 2hr before surgery. Daidzein (Cayman) was dissolved in 100% DMSO (3mg/mL) and final working solution (0.3 mg/mL) was made in 50% DMSO (1:2).

Induction of Sepsis in Mice: Caecal ligation and puncture was produced as described by Wichterman *et al.* (1980). Briefly a 2 cm ventral midline incision was performed on the animals under anaesthesia. Then the caecum was exposed and ligated with 3/0 silk just distal to the ileocecal valve, punctured twice with a 21 gauge needle and returned to the abdomen. The abdominal incision was closed in layers. Normal saline (1 mL/mouse) was given subcutaneously to all mice. Sham-operated mice had undergone the same surgical procedure except caecal ligation and puncture.

Sample collection: Blood was collected from the animals by cardiac puncture at different time intervals –2 and 12 hours in order to establish the time of peak hyperglycemia. Serum was separated by centrifuging the blood at 3500 rpm for 10 minutes and analyzed for glucose and insulin level. Liver was collected and processed for glycogen estimation.

Estimation of Glucose: Glucose was estimated by Glucose Oxidase – Peroxidase (GOD-POD) method using standard kit procured from Span diagnostics, Surat, India.

Liver Glycogen estimation: Glycogen was estimated by using Enzy Chrom ™ glycogen assay kit (BioAssay Systems, USA).

Serum insulin estimation: Serum insulin was estimated by ELISA method using kit procured from Cayman chemicals, USA.

Total RNA isolation: Tissues were collected after 2 h of surgery (sham/sepsis). The heart was isolated in 1% diethyl pyrocarbonate-treated autoclaved phosphate buffer saline. It was cleaned of surrounding adipose tissue and stored in RNA later at -20° C. Total RNA was isolated with Mini RNA Isolation kit as per the manufacturer's instructions. The samples were treated with RNase free-DNase, and the DNase was subsequently inactivated by heating at 56°C for 10 min and immediately chilled to 4°C. The purity of the RNA was checked by A_{260}/A_{280} ratio and quantified as follows 1 OD = 40 μg mL^{-1}.

Quantitative Real-time Polymerase Chain Reaction: Real-time polymerase chain reaction (PCR) was conducted using SYBR Green I master mix (Maxima SYBR Green qPCR Master mix [2X], Fermantas). Each sample was run in duplicate in a 20 μL-reaction. The 20-μL reaction mixtures consisted of 10 μL SYBR Green master mix, 0.4 μL ROX Low, 1.0 μL from 10 pM stock solution of each of the gene-specific forward and reverse primers, 1 μL of cDNA and volume was made up to 20 μL with RNase-free water. The real-time PCR reaction started with initial incubation at 95°C for 10 min followed by 40 cycles of amplification with denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C for 30 s each. The optimum annealing temperatures determined by PCR for the respective gene using the specific primers were as follows: for the insulin receptor gene were F 5'-CAATGGGACCACTGTATGCATCT-3', R 5' -GTCCGGCACGTACACAGAAGA-3' (104 bp) at 57°C; for the *GAPDH* gene were F 5' AACTTTGGC ATTGTGGAAGG3', R 5'ACACATTG GGGGTAGGAACA3' (223 bp) at 57°C. To assess the specificity of the amplified product, a dissociation curve was generated at temperatures of 55°C through 95°C. The result was expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold automatically adjusted by the instrument was used for the generation of C_T values.

To study the relative change in gene expression, the $2^{-ÄÄC}{}_T$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was "fold change = $2^{-ÄÄC}{}_T$," where $ÄÄC_T = (C_{T,\text{target gene}} - C_{T,\text{GAPDH}})$ control - $(C_{T,\text{target gene}} - C_{T,\text{GAPDH}})$ treatment. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene GAPDH to account. For sepsis and atorvastatin + sepsis, mouse evaluation of $2^{-ÄÄC}{}_T$ indicates the fold change in gene expression relative to sham control (i.e., fold change in sham control = 1). The results were analyzed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene.

Statistical analysis

Results were expressed as mean ± S.E. Data were compared by employing one way ANOVA followed by Newman Keuls' post hoc test. p-value of <0.05 was considered as statistically significant.

RESULTS

Effect sepsis on serum glucose level

Figure 1 depicts the effect of sepsis on serum glucose concentration following CLP. Serum glucose level was significantly ($p<0.05$) elevated at 2 h post-CLP (243±17.43 mg/dl) in comparison to the sham at 2 h (117.8±12.05 mg/dl). These findings were indicative of

Fig.1.
Effect of sepsis on serum glucose level in mice at different time interval

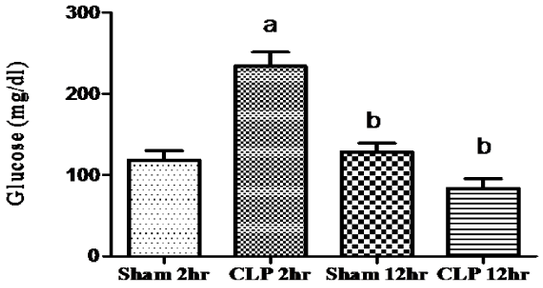


Fig.2.
Effect of daidzein on sepsis -induced alteration in serum glucose level in mice.

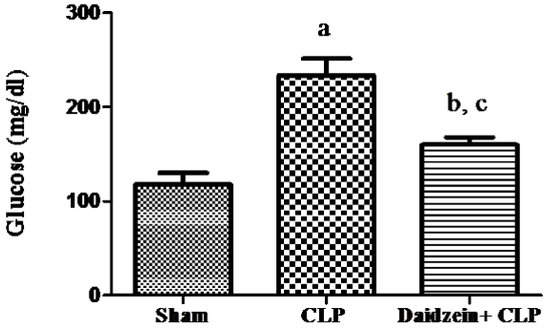
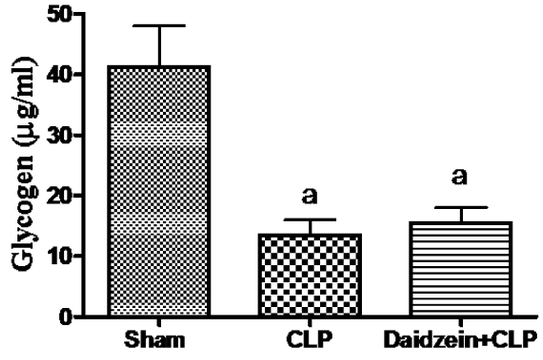


Fig. 3.
Effect of daidzein on sepsis-induced alteration in liver glycogen in mice.



acute hyperdynamic metabolic phase. By 12 h post CLP, serum glucose level was significantly ($p<0.05$) reduced (83.37± 11.65 mg/dl) in comparison to CLP 2h. Based on these results, 2h post CLP had been chosen to determine the other parameters.

Effect of daidzein on sepsis- induced alteration in serum glucose level

Fig4.
Effect of daidzein on the sepsis-induced alteration in serum insulin in mice

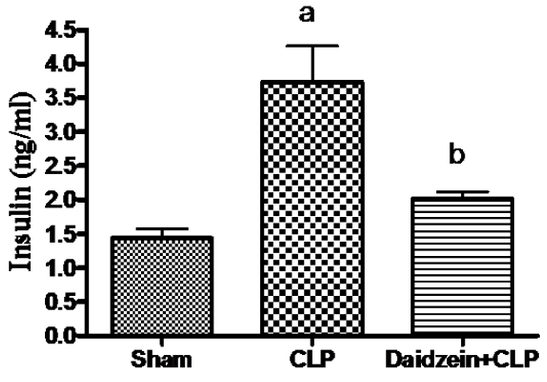
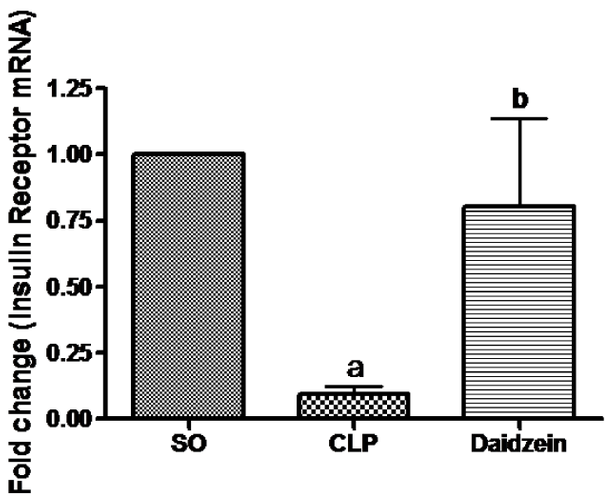


Fig5.
Effect of daidzein on the sepsis-induced alteration in insulin receptor mRNA expression in mice liver tissue.



Serum glucose level was significantly ($p<0.05$) elevated at 2 h post-CLP (243±17.43 mg/dl) in comparison to the sham at 2 h (117.8±12.05 mg/dl). (Figure 2) and daidzein pre-treatment significantly ($p<0.05$) attenuated the sepsis-induced hyperglycemia (160.2±7.69 mg/dl).

Effect of daidzein on sepsis-induced alteration in liver glycogen

In septic animals, liver glycogen level was significantly ($p<0.05$) reduced (13.48±2.49 µg/ml) in comparison to the sham (41.26±6.81µg/ml). However, daidzein pre-treatment had no effect on the liver glycogen level (15.49 ±2.52 µg/ml**)** **(**Figure 3).

Effect of daidzein on sepsis-induced alteration in serum insulin

Sepsis caused significant increase in the serum

insulin level (3.72±0.54 ng/ml) in comparison to the Sham (1.43±0.14 ng/ml). Daidzein pre-treatment showed significant ($p<0.05$) reduction in insulin level (2.01±0.10 ng/ml**) (**Figure 4).

Effect of daidzein on sepsis-induced alteration in liver insulin receptor mRNA expression in mice

Sepsis caused significant reduction in the insulin receptor mRNA expression (0.09±0.02) in comparison to the Sham (1.00). Daidzein pre-treatment showed significant ($p<0.05$) increase in insulin receptor mRNA expression (0.8±0.33**) (**Figure 5).

DISCUSSION

Isoflavones are currently the focus of much attention because epidemiological data strongly suggest that they promote healthy effects in humans. Treatment with isoflavones decreases plasma glucose and insulin levels in rodent models of obesity and insulin-resistance (Gilbert and Liu, 2013; Davis *et al.*, 2007] and improves insulin-sensitivity and glucose uptake in mice (Cederroth *et al.*, 2008, Nordentoft *et al.*, 2008).

In this study, we observed hyperglycemia 2 h post CLP. Hyperglycemia is a cardinal feature of sepsis because of the glycogenolysis by increased plasma catecholamine level. Increase in plasma catecholamine level in sepsis has been shown in a recent work from our laboratory (Thangamalai *et al.*, 2014). Accordingly, significant reduction in liver glycogen content 2 h post CLP was observed. Liver glycogen level is reduced in CLP model of sepsis at 2 h as well as 12 h (Hyde *et al.*, 1990).

Based on our observation of hyperglycemia at 2 h post CLP, we chose this time point for the assessment of the effect of daidzein pre-treatment. Daidzein pre-treatment partially restored sepsis-induced hyperglycemia in this study. However, daidzein was unable to restore the glycogen level in liver. This is an interesting observation as daidzein pre-treatment has reversed the hyperglycaemic condition. To explain this, we measured plasma insulin level and insulin receptor mRNA expression in septic and daidzein pre-treated mice liver. We found a typical situation of insulin resistance 2 h post CLP. There was a significant increase in plasma insulin level in septic mice with concordant hyperglycemia. Fasting insulin levels were shown to be higher in septic rats than in the sham group (Calisto *et al.*, 2010). Similarly, patients with hypermetabolic stress such as sepsis have a significant increase in plasma insulin concentration (Siegel *et al.*, 1979). Moreover, there is a significant reduction in insulin receptor mRNA expression in liver tissue in our study. Reduced insulin receptor mRNA expression indicates reduced insulin sensitivity in the cells leading to insulin resistance. Daidzein pretreatment significantly lowered the plasma insulin level but increased the insulin receptor mRNA expression in liver indicating improved insulin sensitivity in the cells leading to better utilisation of glucose by the cells and preventing hyperglycaemia. Surprisingly, liver glycogen content was not restored by daidzein pretreatment which may be explained by the fact that the glucose in liver cells can be utilized by metabolic pathways other than glycogen synthesis such as increased glycolysis. Increase in glycolysis may improve ATP generation which is impaired in sepsis (Vanasco *et al.*, 2012).

In conclusion, the results of the present study suggest that phytoestrogen, daidzein has the potential to improve insulin resistance in mouse model of sepsis. Nevertheless further studies are needed to elucidate the full potential and mechanism of daidzein in sepsis.

REFERENCES

Calisto, K.L., Carvalho, B.d.M., Ropelle, E.R., Mittestainer, F.C., Camacho, A.C.A. and Dioze, G. (2010) Atorvastatin improves survival in septic rats: effect on tissue inflammatory pathway and on insulin signaling. *PLoS One.* **5**: e14232.

Cederroth, C., Vinciguerra, M., Gjinovci, A., Kühne, F., Klein, M. and Cederroth, M. (2008) Dietary phytoestrogens activate AMP-activated protein kinase with improvement in lipid and glucose metabolism. *Diabetes.* **57**: 1176–85.

Cheong, S.H., Furuhashi, K., Ito, K., Nagaoka, M., Yonezawa, T., Miura, Y. and Yagasaki, K. (2014) Daidzein promotes glucose uptake through glucose transporter 4 translocation to plasma membrane in L6 myocytes and improves glucose homeostasis in Type 2 diabetic model mice. *J. Nutr. Biochem.* **25(2)**: 136-43.

Davis, J., Higginbotham, A., O'Connor, T., Moustaid-Moussa, N., Tebbe, A. and Kim, Y.C. (2007) Soy protein and isoflavones influence adiposity and development of metabolic syndrome in the obese male ZDF rat. *Ann. Nutr. Metab.* **51**: 42–52.

Gariani, K., Drifte, G., Dunn-Siegrist, I., Pugin, J. and Jornayvaz, F. R. (2013) Increased FGF21 plasma levels in humans with sepsis and SIRS. *Endocrine connections.* ***2*(3)**: 146-153.

Gilbert, E.R. and Liu, D. (2013) Anti-diabetic functions of soy isoflavone genistein: mechanisms underlying its effects on pancreatic β-cell function. *Food Funct*., 4(2):200-12.

Goldenberg, N.M., Steinberg, B.E., Slutsky, A.S. and Lee, W.L. (2011) Broken barriers: a new take on sepsis pathogenesis. *Sci. Transl. Med*. **3**: 88ps25-88ps25.

Hyde, S.R., Stith, R.D. and McCallum, R.E**.** (1990) Mortality and bacteriology of sepsis following cecal ligation and puncture in aged mice. *Infect. Immun*. **58:** 619-24.

Kyle, U.G., Bu, J.A.C., Kennedy, C.E. and Jefferson, L.S. (2010) Organ dysfunction is associated with hyperglycemia in critically ill children. *Intensive care Med.* **36**: 312-320.

Lind, L. and Lithell, H. (1994) Impaired glucose and lipid metabolism seen in intensive care patients is related to severity of illness and survival. *Clin. Intensive Care.* **5**: 100-5.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\ddot{A}\ddot{A}ct}$ methods. *Methods.* **25**: 402-408.

Mayr, F.B., Yende, S. and Angus, D.C. (2013) Epidemiology of severe sepsis. *Virulence.* **5(1)**: 1-8.

Mueller, N.T., Odegaard, A.O., Gross, M.D., Koh, W.P., Yu , M.C., Yuan, J.M. and Pereira, M.A. (2011) Soy intake and risk of type 2 diabetes mellitus in Chinese Singaporeans : Soy intake and risk of type 2 diabetes. *Eur. J. Nutr.* **51(8)**: 1033-1040.

Nordentoft, I., Jeppesen, P., Hong, J., Abudula, R. and Hermansen, K. (2008) Increased insulin sensitivity and changes in the expression profile of key insulin regulatory genes and beta cell transcription factors in diabetic KKAy-mice after feeding with a soy bean protein rich diet high in isoflavone content. *J. Agric. Food Chem.* **56**: 4377–85.

Preiser, J.C., Devos, P. and Chiolero, R. (2010) Which factors influence glycemic control in the intensive care unit? *Curr. Opin. Clin. Nutr. Metab. Care*. **13(2)**: 205-10.

Russell, J.A . (2006) Management of sepsis. *N. Engl. J.Med.* **355**: 1699–713.

Siegel, J.H., Cerra, F.B., Coleman, B., Giovannini, I., Shetye, M., Border, J.R. and McMenamy, R.H. (1979) Physiological and metabolic correlations in human sepsis. *Surgery.* **86***:* 163-93.

Terblanche, M., Almog, Y., Rosenson, R.S., Smith, T.S . and Hackam, D.G. (2006) Statins: panacea for sepsis? *Lancet Infect. Dis.* **6**: 242–8.

Thangamalai R, Kandasamy K, Sukumaran SV, Reddy CE, Singh V, Choudhury S, Parida S, Singh TU, Boobalan R and Mishra SK. (2014) Atorvastatin prevents sepsis-induced downregulation of myocardial β_1-adrenoceptors and decreased cAMP response in mice. *Shock. Jan.* **14**. [Epub ahead of print].

Todi, S., Chatterjee, S., Sahu, S. and Bhattacharyya, M. (2010) Epidemiology of severe sepsis in India: an update. *Crit. Care.* **14(Suppl 1)**: 382.

Vanasco, V., Magnani, N.D., Cimolai, M.C., Valdez, L.B., Evelson, P., Boveris, A. and Alvarez, S. (2012). Endotoxemia impairs heart mitochondrial function by decreasing electron transfer, ATP synthesis and ATP content without affecting membrane potential. J *Bioenerg Biomembr.* **44**: 243-52.

Vincent, J.L., Serrano, E.C. and Dimoula, A. (2011) Current management of sepsis in critically ill adult patients. *Expert Rev. Anti. Infect. Ther.* **9**: 847-56.

Wicherman, K.A., Baue A.E. and Chaudry, I.H. (1980) Sepsis and septic shock - A review of laboratory models and a proposal. *J. Surg. Res.* **29**: 189-201.

Received on: 01.12.2013
Accepted on: 22.12.2013