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Acute phase response in experimentally *Escherichia coli* serotype O55:B5 induced endotoxemia and its comparative treatment with dexamethasone and flunixin meglumine in Iranian fat-tailed sheep

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ABSTRACT

This experiment was performed in order to clarify the effect of acute phase response following the induction of endotoxemia by *Escherichia coli* serotype O55:B5 and regarding its comparative treatment with dexamethasone and flunixin meglumine in the Iranian fat-tailed sheep. Fifteen, clinically healthy, one-year old Iranian fat-tailed ewes were randomly assigned into three equal (n = 5) experimental groups, comprising of a control group, and a Dexa and a Flnx group. Lipopolysaccharide from *Escherichia coli* serotype O55:B5 was administered intravenously at 20 µg/kg. All experimental groups underwent intravenous fluid therapy for 120 minutes after lipopolysaccharide injection. 180 minutes after lipopolysaccharide, Dexamethasone (in Dexa group at 1 mg/kg) and flunixin meglumine (in Flnx group at 2.2 mg/kg) were injected, along with the intravenous fluid for 60 minutes. The control group received only lipopolysaccharide and was treated with an intravenous fluid without any drug. The researchers collected blood samples from all the ewes and assayed separated sera for serum acute phase proteins (serum amyloid A and haptoglobin) and inflammatory cytokines (tumor necrosis factor-alpha and interferon-gamma). In all experimental groups there a rapid increase was noted in the amount of acute phase proteins and inflammatory cytokines after endotoxemia induction ($P<0.05$). At the same time, there were no significant differences in the amount of acute phase proteins and inflammatory cytokines concentration for groups treated with dexamethasone and flunixin meglumine ($P>0.05$). The findings also revealed that immediate intravenous administration of dexamethasone and flunixin meglumine reduced and controlled the acute phase response in sheep endotoxemia due to *Escherichia coli* serotype O55:B5.

Key words: endotoxemia, acute phase response, dexamethasone, flunixin meglumine, Iranian fat-tailed sheep

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Introduction

The acute phase response (APR) is a set of immediate host inflammatory reactions that counteract the challenges of endotoxemia. APR isolates and counteracts pathogens, prevents further pathogen entry by minimizing tissue damage and encourages repair processes; it therefore permits host homeostatic mechanisms rapidly to re-establish normal physiological functions (KUSHNER, 1982; BAUMANN and GAULDIE, 1994). APR induces an inflammatory mediator cascade, accompanied by local vascular and systemic multiorgan effects. Systemic multiorgan effects cause biosynthetic changes, prominently in the liver, altering the profile of circulating plasma proteins. One of the most important experiments, which intensively studied systemic responses to an acute inflammatory stimulus, was the study of alterations in the hepatic biosynthetic profile of acute phase proteins (APPs) (STEEL and WHITEHEAD, 1994; GABAY and KUSHNER, 1999). APPs and their changes due to different inflammatory and non-inflammatory conditions have been studied in detail in many animal species (KANEKO, 1997; ECKERSALL, 2000; PETERSEN et al., 2004; MURATA et al., 2004; MURATA, 2007). Inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), are involved in the systemic inflammation and are the members of a molecule group that stimulate the APR. Their sensitivity in the evaluation of APPs and inflammatory cytokines for diagnosis of a disease is higher than hematological and clinical tests. Hematological factors show such great variance in different stages of inflammatory diseases, and clinical tests diagnose the diseases when it has already developed (NAZIFI et al., 2008). Knowledge regarding the amount of APR in sheep undergoing endotoxemia and following its treatment is rare. Therefore, the present experiment was designed to clarify the amount of APR following the induction of endotoxemia by *Escherichia coli* serotype O55:B5 and concerning its comparative treatment with dexamethasone and flunixin meglumine in Iranian fat-tailed sheep.

Materials and methods

Animals. The present experiment was performed after receiving approval from the Ethics Committee of the School of Veterinary Medicine, Shiraz University. Fifteen, clinically healthy, one-year old Iranian fat-tailed ewes (25 ± 1.5 kg bodyweight) were randomly selected in April 2011. All the animals were kept in the Research Barn of the Agricultural College of Shiraz University, Shiraz (latitude 29° 32' N and longitude 52° 35' E, 1810 m above sea level), in the south of Iran. Four weeks before beginning our experiments, each sheep was given albendazole (15 mg/kg, orally; Dieverm®600, Razak Pharmaceutical Co, Tehran, Iran) and ivermectin (0.2 mg/kg, subcutaneously; Erfamectin®1%; Erfan Pharmaceutical Co, Tehran, Iran) in order to control its internal and external probable parasites. All the ewes were kept in open-shed barns, with free

access to water and shade. The rations prepared were mainly alfalfa hay, corn silage, corn and barley. Subsequently, the ewes were assigned randomly into 3 equal experimental (n = 5) groups, that is a Control group, and Dexa and Flnx groups.

Chemicals and drugs. Phenol extracted lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich®; product NO. L2880) was used to induce endotoxemia in ewes at 20 µg/kg. Until it was used, this endotoxin was diluted in sterile phosphate-buffered saline (PBS) and divided into 15 equal doses, each containing 500 µg endotoxin and stored at -80°C. In each experiment, one dose was thawed and infused intravenously as described below. Dexamethasone (Vetacoid® 0.2%, Aburaihan Pharmaceutical Co., Tehran, Iran) and flunixin meglumine (Meganix® 5%, Erfan Pharmaceutical Co., Tehran, Iran) were infused intravenously as described in the experimental procedures. The intravenous fluid used in this experiment was dextrose 5% plus sodium chloride 0.45% (Shahid Ghazi Pharmaceutical CO., Tabriz, Iran).

Experimental procedures

Induction and treatment of endotoxemia. The schematic diagram of our experimental design is represented in Fig. 1. A 16 gauge 5.1 cm catheter was secured in the left jugular vein and used for blood sampling, endotoxin and drug infusions. All fifteen ewes were examined clinically before the LSP injection and 1, 2, 3, 4, 5, 6 and 24 hours after it. The clinical parameters monitored during experiment include: rectal temperature, heart and respiratory rates, mucous membrane color, capillary refill time, appetite and fecal consistency. Thawed LPS (500 µg) was diluted in 250 milliliters of normal saline and infused intravenously at the rate of 10 mL/kg/hr. After LPS injection, intravenous fluid therapy was performed for all experimental groups over 120 minutes, by dextrose 5% plus sodium chloride 0.45% at the rate of 20 mL/kg/hr. Dexamethasone (in Dexa group at 1 mg/kg) or flunixin meglumine (in Flnx group at 2.2 mg/kg) was used at 180 minutes after LPS injection, along with the intravenous fluid over 60 minutes. The control group received LPS and was treated only with intravenous fluid without any drugs. To prevent hypoglycemia, blood glucose was monitored in all animals, using a rapid response glucose meter device (Accucheck Active®, Roche, Germany).

Blood sampling and serological assays. Blood samples were collected from all ewes through the fixed catheter before and 1, 2, 3, 4, 5, 6 and 24 hours after LPS injection in plain tubes. Immediately after collection, sera were separated by centrifugation (for 10 minutes at 3,000×g) and stored at -22 °C until assayed.

Haptoglobin (Hp) was measured according to prevention of hemoglobin peroxidase activity, which is directly proportional to the amount of Hp. The analytical sensitivity of this test in serum has been determined as 0.0156 mg/mL for Hp by the manufacturer (Tridelta Development Plc, Wicklow, Ireland). Serum amyloid A (SAA) was measured by a solid phase sandwich ELISA. The analytical sensitivity of this test in serum has

been determined as 0.3 μg/mL for SAA by the manufacturer (Tridelta Development Plc, Wicklow, Ireland). TNF-α and IFN-γ were also measured by a solid phase sandwich ELISA (AbC 606 and AbC 607, respectively; Votre fournisseur AbCys S.A. Paris, France).

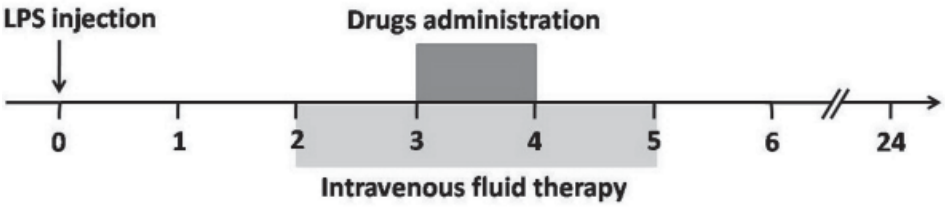

Fig. 1. Schematic diagram of the present experimental design. Lipopolysaccharide (LPS) was injected at hour zero and intravenous fluid therapy was commenced 2 hrs later in Iranian fat tailed sheep. Dexamethasone and flunixin meglumine were infused via fluid between hours 3 and 4, over 60 minutes in the Dexa and Flnx groups, respectively. Venous blood sampling was performed at all demonstrated times.

Statistical analyses. Data were described as mean ± standard error of mean (SEM). We performed statistical analysis using one-way ANOVA, with an LSD post-hoc test to compare mean concentrations of the different serological factors between the different experimental groups, within similar times. The paired sample t-test was used to determine differences between the experimental groups at two different times, using SPSS software (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). The level of significance was set at $P<0.05$.

Results

After endotoxemia induction, SAA and Hp elevated rapidly in both experimental groups (Fig. 2). These rapid elevations differed between hour zero and hour 3 (after which intravenous fluid therapy commenced; $P<0.05$). For the control group, serum concentrations of SAA at hours 6 and 24 was higher than the other two experimental groups at the same hours ($P<0.05$; Fig. 2A). In the control group, serum Hp concentrations at hours 4, 5, 6 and 24 hours were significantly higher than in the Dexa and Flnx groups at the same times ($P<0.05$; Fig. 2B). There were no significant differences seen between serum APP concentrations in the Dexa and Flnx groups at similar times ($P>0.05$). There was also no significant difference seen in the concentrations of different factors between hours zero and 24 in either Dexa or the Flx group ($P>0.05$).

A rapid elevation of serum TNF-α and IFN-γ was detected after endotoxemia induction in the experimental groups (Fig. 3). There were significant differences between the elevation of TNF-α at hour 3 and its base line levels at hour zero ($P<0.05$; Fig. 3A). The same pattern was observed for IFN-γ, also. After hour 4, serum TNF-α and IFN-γ

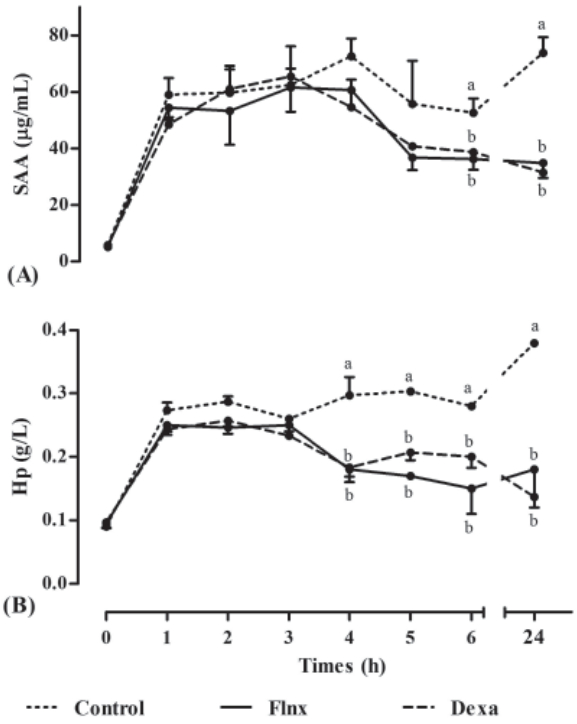

Fig. 2. Serum amyloid A (SAA, A) and haptoglobin (Hp, B) alterations due to experimental endotoxemia in 3 experimental groups of Iranian fat-tailed sheep (n = 5). [a, b] different letters show significant differences in similar hrs between groups (P<0.05).

concentrations in the control group were significantly higher than in the Dexa and Flnx groups (P<0.05). There no significant differences were seen between serum inflammatory cytokine concentrations in the Dexa and Flnx groups at the same hours (P>0.05). Serum concentrations of TNF-α and IFN-γ at hour 24 were not significantly different from the base line levels at hour zero in both treatment groups (P>0.05).

All sheep were alive and healthy after all experiments.

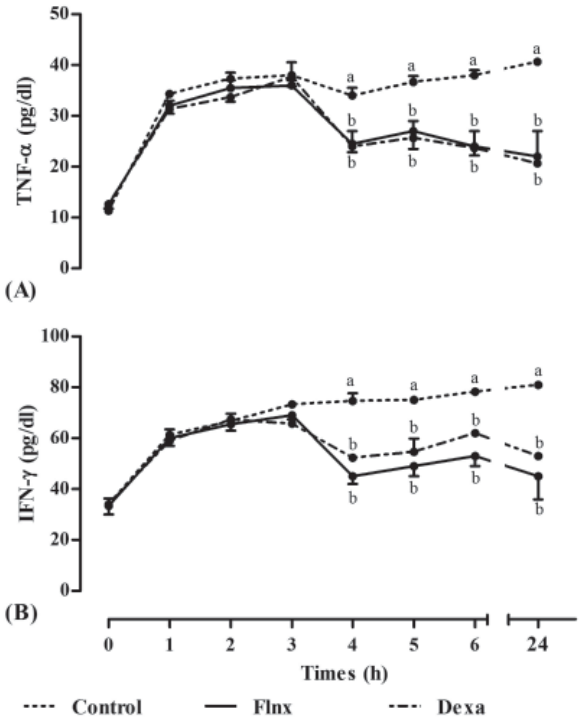

Fig. 3. Tumor necrosis factor-alpha (TNF-α, A) and interferon-gamma (IFN-γ, B) alterations due to experimental endotoxemia in 3 experimental groups of Iranian fat-tailed sheep. [a, b] different letters show significant differences at similar hrs between groups ($P<0.05$).

Discussion

APPs are a group of blood proteins whose concentrations change in animals subjected to external or internal challenges, such as infection, inflammation, surgical trauma or stress (MURATA et al., 2004; GRUYS et al., 2005). APPs and their alterations due to different inflammatory and non inflammatory conditions have been studied intensively in many animal species (MURATA et al., 2004; MURATA, 2007). No study was found in literature regarding the APP profile in endotoxemia due to *Escherichia coli* serotype O55:B5 and its treatment in Iranian fat-tailed sheep.

SAA and Hp, as well as other APPs, have been suggested as markers of stress in animals (ARTHINGTON et al., 2003; HICKEY et al., 2003; PINEIRO et al., 2007). SAA is an apolipoprotein of high-density lipoprotein and is considered to be one of the major APPs in vertebrae (NAKAYAMA et al., 1993; GRUYS et al., 1994; HUSBY et al., 1994). Determination and evaluation of SAA has indicated that this APP could be a valuable factor in the diagnosis of infections (ALSEMGEEST et al., 1994). LEHTOLAINEN et al. (2004) described that during experimental endotoxin-induced mastitis in cattle, SAA concentrations increased both in serum and milk. The results of this experiment showed that SAA elevated rapidly and significantly after endotoxemia induction in all experimental groups ($P<0.05$; Fig. 2A). SAA reflected the course of inflammation and its level correlated with the clinical severity of the inflammation. SAA has the greatest function in bacterial and pyogenic infections, and increases in common infectious diseases (NAZIFI et al., 2008). Elevated serum SAA levels have been found following inflammation and also under conditions unrelated to inflammation, such as physical stress (MURATA et al., 2004). LOMBORG et al. (2008) demonstrated marked SAA responses in healthy adult cattle after they were subjected to complex stressors (transportation, tie stall housing, slippery floors, and social isolation). At the beginning of inflammatory reactions or after an injury, SAA concentrations increase rapidly. However, the underlying mechanism that causes the increase in SAA has not been clearly defined (NAZIFI et al., 2008). Although the serum concentrations of SAA decreased significantly in the Flnx and Dexa groups ($P<0.05$), we found no significant differences between the efficacy of dexamethasone and flunixin meglumine in decreasing SAA at similar times.

Hp is an alpha2-globulin synthesized in the liver and another major APP in numerous species of productive and companion animals (FELDMAN et al., 2000). In ruminants, the level of circulating Hp is negligible in normal animals, but increases over 100-fold with immune stimulation (CONNER et al., 1989). Many studies have indicated the significance of Hp as a clinically useful parameter for evaluation of the occurrence and severity of inflammatory diseases in sheep (PFEFFER and ROGERS, 1989; SKINNER and ROBERTS, 1994). Determination and evaluation of serum Hp have shown that this protein could be valuable in the diagnosis of infection and inflammatory conditions. Serum Hp is most helpful when used together with herd history, clinical signs and laboratory tests (ALSEMGEEST et al., 1994). In the present experiment, following intravenous endotoxin infusion, rapid and significant elevation in serum concentrations of Hp was observed in all ewes ($P<0.05$; Fig. 2B).

TNF-α is a member of a group of cytokines that stimulate the APR, and it is involved in systemic inflammation. The primary role of TNF-α is regulation of immune cells. TNF-α is able to induce apoptotic cell death and inflammation, and to inhibit tumorigenesis and viral replication. It is produced by a broad variety of cell types, including lymphoid cells,

mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. A large amount of TNF-α is released in response to endotoxins. In the liver, TNF-α stimulates APR, leading to an increase in APPs. The responses of endothelial cells to TNF-α include: structural rearrangement, production of neutrophil adherence proteins, expression of procoagulant activity, and down-regulation of thrornbomodulin (MOVAT, 1987). TNF-α causes endothelial dysfunction and apoptosis, triggers procoagulant activity and fibrin deposition, and enhances nitric oxide synthesis in a variety of cells (SENGUPTA et al., 2008). Contributing to the inflammatory changes that occur in many tissues during endotoxemia, neutrophils are activated and stimulated to adhere to the vascular endothelium by TNF-α (SATOMI et al., 1985). TNF-α has especially been amply involved in deleterious host responses (HEINZEL, 1990). Significant ($P<0.05$) and rapid elevation of serum TNF-α was observed before commencing intravenous fluid therapy in both the experimental groups. In the present experimental endotoxemia induction, marked and significant depressions of TNF-α were observed in the Dexa and Flnx groups. No significant differences were seen between dexamethasone and flunixin meglumine in controlling elevated serum TNF-α and the two drugs clearly attenuated the APR, due to decreasing of TNF-α concentrations.

IFN-γ is a dimerized soluble cytokine, that is the only member of the type II class of interferons. IFN-γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections. The importance of IFN-γ in the immune system stems from its ability to inhibit viral replication directly, and, most importantly, its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer and natural killer T cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte effector T cells, once antigen-specific immunity develops. Endotoxin activates macrophage microbicidal effectors functions and stimulates the production of proinflammatory cytokines, such as IFN-γ (SCHRODER et al., 2004). In the present experiment, the results of the IFN-γ assay showed that the value of serum concentrations of this inflammatory cytokine in the control group was significantly higher than in the experimental groups after treatment ($P<0.05$; Fig. 3B). According to our findings, dexamethasone and flunixin meglumine efficiently controlled the serum IFN-γ levels and there was no difference in the potency of the two drugs in this respect.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used in general for the treatment of endotoxemia, because of their analgesic, anti-inflammatory and antipyretic properties. NSAIDs overcome the production of cytokines and reduce the acute hemodynamic response to endotoxemia. Flunixin meglumine is the most commonly used type of NSAID in the treatment of endotoxemia in horses and cattle, and has remained the best choice for treatment of this condition. Flunixin meglumine also adjusts acute

hemodynamic changes commonly seen during endotoxemia, which may increase survival rates (RADOSTITS et al., 2007). The results of the present experiment showed that intravenous administration of flunixin meglumine reduced the serum concentrations of APPs and inflammatory cytokines, to near base line levels within 24 hours (Fig. 2). According to these findings, it may be said that flunixin meglumine can control APR in experimental endotoxemia induced by phenol extracted LPS from *Escherichia coli* serotype O55:B5 in Iranian fat-tailed sheep.

Corticosteroids are used extensively for the treatment of endotoxemia and endotoxic shock. These drugs are less often administered to endotoxemic animals, as a result of a number of studies supporting the use of NSAIDs. Corticosteroids promote capillary endothelial integrity and tissue perfusion, decrease the activation of the complement and clotting cascade, decrease neutrophil aggregation, stabilize lysosomal membranes, protect against hepatic injury and improve survival rate. However, there are concerns about their use in septicemic animals, because they may cause immunosuppression (RADOSTITS et al., 2007). Dexamethasone is the most commonly used corticosteroid in endotoxemia. However, a review of literature revealed that corticosteroids are the most helpful therapeutically when given as a pretreatment in experimental endotoxemia situations (REYNOLDS et al., 2002). It is currently believed that corticosteroids, if they are to be clinically effective, must be given as early as possible to endotoxemic animals (RADOSTITS et al., 2007). Our findings revealed that intravenous administration of dexamethasone, after endotoxemia induction by *Escherichia coli* serotype O55:B5 and the appearance of APR, was effective to reduce serum concentrations of APPs and inflammatory cytokines.

It was concluded that immediate intravenous administration of dexamethasone and flunixin meglumine, at 1 and 2.2 mg/kg, respectively, reduced and controlled APR in *Escherichia coli* serotype O55:B5 endotoxemic sheep. It was also concluded that there were no significant differences between the efficacy of dexamethasone and flunixin meglumine in combating APR due to endotoxemia in Iranian fat-tailed sheep. Furthermore, with sheep as an experimental model, these results may also be extended to other domestic ruminants.

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SAŽETAK

Istraživanje je poduzeto s ciljem da se razjasni učinak odgovora proteina akutne faze nakon pokusno izazvane endotoksemije serovarom O55:B5 bakterije *E. coli* s obzirom na mogućnost njezina liječenja istodobno deksametazonom i fluniksin megluminom u iranske masnorepe ovce. U pokus je bilo uzeto 15 klinički zdravih jednogodišnjh iranskih masnorepih ovaca, koje su bile nasumce podijeljene u tri skupine po pet, tj. kontrolnu te u pokusnu skupinu koja je dobivala deksametazon i skupinu koja je dobivala fluniksin. Lipopolisaharid serovara O55:B5 bakterije *Escherichia coli* bio je primijenjen intravenski u količini 20 µg/kg. Sve životinje pokusnih skupina intravenski su dobile fiziološku otopinu tijekom 120 minuta nakon davanja lipopolisaharida. 180 minuta nakon davanja lipopolisaharida jednoj je pokusnoj skupini primijenjen deksametazon (1 mg/kg), a drugoj fluniksin meglumin (2,2 mg/kg) intravenski u fiziološkoj otopini tijekom 60 minuta. Ovcama kontrolne skupine bio je primijenjen samo lipopolisaharid i fiziološka otopina primijenjena intravenski. Uzorci krvi bili su uzeti od svih ovaca, a serum je bio pretražen na proteine akutne faze (serumski amiloid A i haptoglobin) i upalne citokine (faktor tumorske nekroze alfa i interferon gama). U pokusnim skupinama ustanovljeno je naglo povećanje količine proteina akutne faze i upalnih citokina nakon izazivanja endotoksemije ($P<0,05$). Istodobno nije bila zabilježena značajna razlika u količini proteina akutne faze i koncentracije upalnih citokina za skupine koje su dobile deksametazon i fluniksin meglumin ($P>0,05$). Rezultati su također pokazali da je brza intravenska primjena deksametazona i fluniksin meglumina oslabila odgovor proteina akutne faze kod endotoksemije ovaca izazvane serovarom O55:B5 bakterije *Escherichia coli*.

Ključne riječi: endotoksemija, odgovor akutne faze, deksametazon, fluniksin meglumin, iranska masnorepa ovca