The relationships among acute phase response proteins, cytokines, and enzymes during ovine experimental endotoxemia

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Abstract:

BACKGROUND: The acute phase response is beneficial to the animal in restoring homeostasis, and measuring the circulating acute phase proteins, cytokines, and enzymes can be used to evaluate the innate immune system's responses to invader agents such as bacterial lipopolysaccharide. Measurement of these parameters has shown to be useful as diagnostic and prognostic markers in animal endotoxemia. **OBJECTIVES:** The aim of the present experimental study was expression of the acute phase response following the induction of endotoxemia by *Escherichia coli* serotype O55:B5 in sheep and the relationships among the acute phase response parameters during endotoxemia and their changing patterns. **METHODS:** Five clinically healthy 1-year-old Iranian fat-tailed ewes (25±1.5 kg, bodyweight) were randomly selected and lipopolysaccharide from *Escherichia coli* serotype O55:B5 was infused at 20 µg/kg intravenously to each animal. Fluid therapy was performed in all ewes over 120 minutes after lipopolysaccharide injection and continued for 180 minutes. Blood samples were collected from all ewes prior and 1, 2, 3, 4, 5, 6, and 24 hours after lipopolysaccharide injection and sera were separated. Serum concentrations of haptoglobin, serum amyloid A, tumor necrosis factor-alpha, interferon-gamma, superoxide dismutase, and glutathione peroxidase were assayed. **RESULTS:** The rapid and significant elevation of haptoglobin, serum amyloid A, tumor necrosis factor-alpha, and interferon-gamma were seen after endotoxemia induction. Serum concentrations of superoxide dismutase and glutathione peroxidase were significantly decreased after intravenous lipopolysaccharide infusion. The results of the present experimental study showed that haptoglobin, serum amyloid A, tumor necrosis factor-alpha, and interferon-gamma at all hours studied after endotoxemia induction were positively correlated together. These parameters were negatively correlated with superoxide dismutase and glutathione peroxidase at all hours after lipopolysaccharide infusion. **CONCLUSIONS:** The results of the present experiment can provide evidence for associations among acute phase proteins, cytokines, and enzymes and their changes during endotoxemia in sheep.

Introduction

The acute phase response (APR) is a systemic first defense to tissue injuries, infectious agents, stress, and inflammatory processes. It is characterized by a local reaction at the site of injury followed by a number of systemic process including changes in the concentration of circulating APR proteins, cytokines, and enzymes (Gabay and Kushner, 1999). The APR is beneficial to the animal in restoring homeostasis, and the response is tightly controlled by negative feedback loops. Measuring the circulating APR proteins, cytokines, and enzymes can be used to evaluate the innate immune system's responses to invader agents such as bacterial lipopolysaccharide (LPS) (Eckersall and Bell, 2010). Bacterial LPS from the cell wall of Gram-negative bacteria causes endotoxemia which is considered the cause of most of the pathophysiological reactions. The severity of clinical signs during endotoxemia is considered to depend mainly on the APR (Burvenich et al., 2003). LPS triggers formation of acute phase proteins (APPs), proinflammatory cytokines, and anti-oxidative enzymes produced predominantly by hepatocytes, monocytes, and macrophages (Persson et al., 2003). Measurements of these proteins, cytokines, and enzymes have shown to be useful as diagnostic and prognostic markers in endotoxemia. Furthermore, they can be used in order to assess host defense, tissue damage and response to treatment (Eckersall and Bell, 2010).

There are several studies on drug efficacies on the experimental endotoxemia in sheep (Pfeffer and Rogers, 1989; Chalmeh et al., 2012a, 2012b); however, knowledge regarding the APR undergoing endotoxemia is rare in this breed. Therefore, the present experimental study was designed to clarify and study the APR following the induction of endotoxemia by using *Escherichia coli* serotype O55:B5 based on measurements of circulating APPs (serum amyloid A and haptoglobin), inflammatory cytokines (tumor necrosis factor-alpha and interferon-gamma), and anti-oxidant enzymes (superoxide dismutase and glutathione peroxidase) in Iranian fat-tailed sheep. The results of the present study also reveal the relationship among these parameters during endotoxemia and their changing patterns.

Materials and Methods

Animals: The present experiment was performed after being approved by the Ethics Committee of School of Veterinary Medicine, Shiraz University. Five clinically healthy 1-year-old Iranian fat-tailed ewes (25±1.5 kg, bodyweight) were randomly selected for the project in April 2011. All animals were maintained in Laboratory Teaching Barn of Agricultural College of Shiraz University, Badjgah region (latitude of 29° 32' N and longitude 52° 35' E, 1810 m above sea level), south of Iran. Four weeks before commencing the experiments, each sheep received 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) to control internal and external probable parasites. All ewes were maintained in open-shed barns with free access to water and shade. The ration included mainly alfalfa hay, corn silage, corn, and barley.

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. This endotoxin was diluted in sterile phosphate-buffered saline (PBS) and divided into 5 equal doses, each containing 500 µg endotoxin and stored at -80°C until endotoxemia induction. For each animal, each dose was thawed and infused intravenously as described below. The intravenous fluid used in the present 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 the present experimental design is represented in figure 1. A 16 gauge 5.1 Cm catheter was secured in the left jugular vein and used for blood samplings, endotoxin and fluids infusions. All 5 ewes were evaluated clinically before and 1, 2, 3, 4, 5, 6, and 24 hours after LPS injection. Clinical parameters monitored during experiments included rectal temperature, heart and respiratory rates, mucous membrane color, capillary refill time, appetite, and fecal consistency. Thawed LPS was diluted in 250 milliliter of normal saline and infused intravenously

at the rate of 10 mL/kg/hour. Fluid therapy was performed in all animals over 120 minutes after LPS injection by dextrose 5% plus sodium chloride 0.45% at the rate of 20 mL/kg/hour.

Blood sampling and serological assays: Blood samples were collected from all ewes through the fixed catheter prior and 1, 2, 3, 4, 5, 6, and 24 hours after LPS injection in plain tubes. Immediately after collections, 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 the prevention of peroxidase activity of hemoglobin, which is directly proportional to the amount of Hp (Tridelta Development Plc, Wicklow, Ireland). Serum amyloid A (SAA) was measured by a solid phase sandwich ELISA (Tridelta Development Plc, Wicklow, Ireland). Tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were measured by a solid phase sandwich ELISA (AbC 606 and AbC 607, respectively; Votre fournisseur AbCys S.A. Paris, France). The superoxide dismutase (SOD) activity was measured by a modified method of iodophenyl nitrophenol phenyltetrazolium chloride (Feldman et al., 2000) (RANSOD Kit, Randox Com, UK). The glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine (1967) (RANSEL kit, Randox Com, UK).

Statistical analyses: Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Pearson's correlation test to detect the relationship among studied parameters at each hour, separately, using SPSS software (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). In the present study, the Pearson's correlation coefficient greater than 0.8 was considered as strong, whereas a coefficient lesser than 0.5 described as weak. Paired samples t-test was used to determine differences between two different times (hours zero and 24) during the current experimental study. The level of significance was set at $p<0.05$.

Results

Alterations of SAA, Hp, TNF-α, IFN-γ, SOD, and GPx in different hours during experimental endotoxemia in Iranian fat-tailed sheep are presented in Table 1 in which baseline levels are presented at hour zero. The rapid and significant elevation of SAA, Hp, TNF-α, and IFN-γ was seen after endotoxemia induction ($p<0.05$; Table 1). Serum concentrations of SOD and GPx were significantly decreased after intravenous LPS infusion ($p<0.05$; Table 1). The results of paired samples t-test showed that amounts of SAA, Hp, TNF-α, and IFN-γ at 24th hour were significantly higher than baseline values at hour zero and the serum activities of SOD and GPx at hour 24 were significantly lower than normal levels ($p<0.05$).

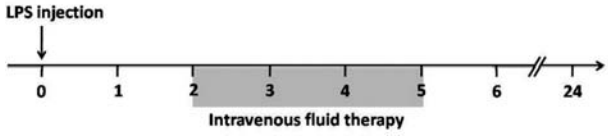

Figure 1. Schematic diagram of the present experimental design. Lipopolysaccharide (LPS) was injected at zero hour, and intravenous fluid therapy was commenced 2 hours later in Iranian fat tailed sheep and continued for 180 minutes. Venous blood sampling was performed in all demonstrated hours.

The correlations among SAA, Hp, TNF-α, IFN-γ, SOD and GPx prior and 1, 2, 3, 4, 5, 6, and 24 hours after LPS injection are presented in Table 2. There were no significant correlations ($p>0.05$) before endotoxemia induction (Table 2). The results of the present experimental study showed that SAA, Hp, TNF-α, and IFN-γ at all hours after endotoxemia induction were positively correlated together (Table 2). These parameters were negatively correlated with SOD and GPx at all hours after LPS infusion. The correlation coefficients were positive among serum activities of anti-oxidative enzymes (SOD and GPx) at all hours during endotoxemia (Table 2).

All sheep were permanent survivors, alive, and healthy after all experiments.

Discussion

The APR is part of the early-defense or innate

Table 1. Alterations (Mean±SD) of acute phase response parameters in different hours of experimental endotoxemia in Iranian fat-tailed sheep (n=5). Serum amyloid A: SAA; Haptoglobin: Hp; Tumor necrosis factor-alpha: TNF-α; Interferon-gamma: IFN-γ; Super oxide dismutase: SOD; Gluthatione peroxidase: GPx.

Hours	SAA (µg/mL)	Hp (g/L)	TNF-α (pg/dL)	IFN-γ (pg/dL)	SOD (U/g Hp)	GPx (U/g Hp)
0	5.82±1.13	0.09±0.01	11.33±0.57	34.00±4.00	1023.33±27.93	196.00±4.58
1	59.08±10.14	0.27±0.02	34.33±1.15	61.33±2.08	616.66±12.75	146.66±1.52
2	59.69±10.61	0.30±0.01	37.33±2.08	66.66±2.51	742.33±28.31	150.33±8.14
3	62.47±11.76	0.26±0.01	38.00±4.35	73.33±2.08	637.66±23.18	145.66±6.42
4	72.59±11.04	0.29±0.05	34.00±2.64	65.66±5.13	661.66±23.26	142.00±6.08
5	50.80±12.39	0.30±0.00	36.66±2.08	75.00±2.00	629.33±29.93	153.66±6.80
6	52.61±8.88	0.28±0.01	38.00±1.73	78.33±1.15	612.00±18.19	145.33±8.38
24	73.86±9.62	0.38±0.01	40.66±0.57	81.00±1.00	593.00±14.00	140.66±4.93

Table 2. Correlations among acute phase response parameters in Iranian fat-tailed sheep with experimentally induced endotoxemia. Significant correlations are indicated by star ($p<0.05$).

		SAA (µg/mL)	Hp (g/L)	TNF-α (pg/dL)	IFN-γ (pg/dL)	SOD (U/g Hp)
Base line (Hour 0)	Hp (g/L)	-0.964				
	TNF-α (pg/dL)	-0.968	0.866			
	IFN-γ (pg/dL)	0.712	-0.500	-0.866		
	SOD (U/g Hp)	0.996	-0.984	-0.940	0.644	
	GPx (U/g Hp)	-0.065	0.327	-0.189	0.655	-0.156
Hour 1	Hp (g/L)	0.949				
	TNF-α (pg/dL)	0.846	0.971			
	IFN-γ (pg/dL)	0.747	0.500	0.277		
	SOD (U/g Hp)	-0.901	-0.992*	-0.993*	-0.386	
	GPx (U/g Hp)	0.974	-0.996*	0.945	-0.577	0.976
Hour 2	Hp (g/L)	0.225				
	TNF-α (pg/dL)	0.135	0.996*			
	IFN-γ (pg/dL)	0.507	0.954	0.923		
	SOD (U/g Hp)	-0.226	-1.000*	-0.996*	-0.954	
	GPx (U/g Hp)	-0.874	-0.670	-0.600	-0.862	0.671
Hour 3	Hp (g/L)	0.734				
	TNF-α (pg/dL)	0.185	0.803			
	IFN-γ (pg/dL)	1.000*	0.721	0.165		
	SOD (U/g Hp)	-0.098	-0.604	-0.960*	-0.117	
	GPx (U/g Hp)	-0.929	-0.933*	-0.535	-0.922	0.277
Hour 4	Hp (g/L)	0.727				
	TNF-α (pg/dL)	0.437	0.300			
	IFN-γ (pg/dL)	0.108	0.761	0.847		
	SOD (U/g Hp)	-0.556	-0.975*	-0.504	-0.886	
	GPx (U/g Hp)	-0.999*	-0.751	-0.404	-0.144	0.586
Hour 5	Hp (g/L)	0.586				
	TNF-α (pg/dL)	0.375	0.971*			
	IFN-γ (pg/dL)	0.913	0.866	0.721		
	SOD (U/g Hp)	-0.955*	-0.800	-0.632	-0.993*	
	GPx (U/g Hp)	-0.948*	-0.297	-0.059	-0.735	0.811
Hour 6	Hp (g/L)	0.850				
	TNF-α (pg/dL)	0.526	0.000			
	IFN-γ (pg/dL)	1.000*	0.866	0.500		
	SOD (U/g Hp)	-0.497	-0.879	-0.476	-0.524	
	GPx (U/g Hp)	-1.000*	-0.835	-0.551	-0.998*	0.472
Hour 24	Hp (g/L)	0.854				
	TNF-α (pg/dL)	0.521	0.000			
	IFN-γ (pg/dL)	0.854	1.000*	0.000		
	SOD (U/g Hp)	-0.393	-0.143	-0.990*	-0.143	
	GPx (U/g Hp)	-0.566	-0.912*	-0.410	-0.912*	0.536

immune system, which is triggered by different stimuli including trauma, infection, stress, neoplasia, and inflammation. The APR results in a complex systemic reaction with the goal of reestablishing homeostasis and promoting healing (Murata et al., 2004). Innate immune responses during APR are

dependent on cytokines and chemokines, which are generated by activated cells including monocytes, macrophages, fibroblasts, endothelium, platelets, keratinocytes, and T cells (Janeway et al., 2001). These proinflammatory signals have numerous effects throughout the body including the APR. The APR is a core part of the innate immune response and is observed across all animal species (Murata et al., 2004).

APPs are blood proteins primarily synthesized by hepatocytes and other organs, such as mammary glands as well, as part of the APR. APPs have been well recognized for their application to veterinary diagnostic medicine and have been described to have value in the diagnosis and prognosis of the diseases (Conner et al., 1989; Pfeffer and Rogers, 1989). Like human APPs, animal APPs have been well documented to be sensitive to similar triggering events. In large animals, APPs have been further proposed as markers of herd health (Murata et al., 2004). The biological functions of APPs and their diagnostic and prognostic values are vast and have been reviewed (Cray et al., 2009).

SAA, often classified as a major APP in ruminants, has been demonstrated to result in chemotaxis of monocytes, polymorphonuclear cells, and T cells. In addition, SAA has marked inhibitory effects and is assumed to be important in the down regulation of the inflammatory process (Gruys et al., 1994). Hp reduces oxidative damage associated with hemolysis by binding free hemoglobin. In addition, it has been observed to have bacteriostatic and immunomodulatory effects (Feldman et al., 2000).

The results of the present study showed that serum concentrations of SAA and Hp significantly increased after endotoxemia induction ($p<0.05$), and the highest amounts of these APPs were seen at 24^{th} hour of this experiment (Table 1). SAA and Hp were positively correlated with TNF-α and IFN-γ at all hours after endotoxemia induction which indicated that changing the circulating values of SAA and Hp has direct relation to alterations of TNF-α and IFN-γ during endotoxemia. Unlike theses relationships, the correlations among APPs and activities of SOD and GPx were negative after endotoxemia induction which reveals the indirect relations among these APPs and anti-oxidant enzymes following APR during endotoxemia (Table 2).

TNF-α is a cytokine involved in systemic inflammation and a member of a group of cytokines that stimulate the APR. Large amounts of TNF-α are released in response to endotoxins. On the liver, TNF-α stimulates the APR, leading to an increase in APPs. TNF-α in particular has been amply implicated in deleterious host responses (Heinzel, 1990). 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. Endotoxin activates macrophage microbicidal effector functions and production of proinflammatory cytokines, such as IFN-γ (Schroder et al., 2004). The pathology of endotoxemia is probably jointly mediated by multiple cytokines released during sepsis. Both exogenously administered and endogenously produced IFN-γ demonstrably contribute to endotoxic mortality as well (Heinzel, 1990). The ability of IFN-γ to increase macrophage TNF-α production by both transcriptional and translational mechanisms has been well described (Burchett et al., 1988).

According to our findings, serum concentrations of TNF-α and IFN-γ significantly increased after endotoxemia induction ($p<0.05$) and their highest amounts were detected 24 hours after endotoxemia induction (Table 1). Based on the positive correlations among these inflammatory cytokines and APPs at all hours after endotoxemia induction in this study, it can be mentioned that relations of TNF-α and IFN-γ are direct to SAA and Hp during APR following induction of endotoxemia. The results of the present study showed that serum concentrations of TNF-α and IFN-γ had indirect relationship to serum SOD and GPx activities.

Superoxide is one of the common reactive oxygen species in the cell, and SOD is the main antioxidant enzyme which can scavenge it. Superoxide radicals release during the chemical reactions as part of the various metabolic pathways, and SOD is responsible for the quenching these radicals. SOD can protect the O2 metabolizing cells against the harmful effects of the superoxide by catalyzing the O2 free radicals. Hence, the activity and level of SOD can demonstrate the severity of infectious and non-infectious stressful conditions (Bauer and Bauer, 1999).

GPx like as SOD is an antioxidant enzyme which

protects cells from the harmful effects of free radicals (Pompella et al., 2003). GPx is found commonly in its reduced form, and glutathione reductase reverts it to active against free radicals (Pastore et al., 2003). Determining the activity of GPx can be used as an indicator for oxidative stress conditions (Podil'chalk et al., 1996).

The results of the SOD and GPx assays showed that serum activities of these anti-oxidant enzymes significantly depressed after endotoxemia induction ($p<0.05$), and their lowest activities were detected 24 hours after endotoxemia induction (Table 1). According to these results, it may be suggested that the changing pattern of anti-oxidant enzyme activities are consumptive during APR following endotoxemia induction. The results of the Pearson's correlation test showed that serum activities of SOD and GPx in this experimental study were negatively correlated with APPs and inflammatory cytokines which reveals the indirect relations among these factors and anti-oxidant enzymes during the endotoxemia (Table 2).

In conclusion, it can be mentioned that APR triggers immediately after *Escherichia coli* serotype O55:B5 endotoxemia induction in the Iranian fat-tailed sheep. In the present experiment, we have shown that synthesis and releasing the APPs and inflammatory cytokines increased during APR following endotoxemia, and serum activities of anti-oxidant enzymes decreased. Furthermore, our results provide evidence for associations among APPs, inflammatory cytokines, and anti-oxidant enzymes and their changes during endotoxemia in a small ruminant model.

Conflict of interest statement: The authors have declared no conflicts of interest.

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ارتباط بین پروتئین‌ها، سیتوکین‌ها و آنزیم‌های پاسخ فاز حاد در خلال یک مدل تجربی اندوتوکسمی در گوسفند

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چکیده

زمینه مطالعه: پاسخ فاز حاد از دیدگاه حفظ هموستاز بدن حیوان حائز اهمیت است و سنجش پروتئین ها، سیتوکین ها و آنزیم‌های فاز حاد موجود در گردش خون را می توان در ارزیابی عملکرد سطح ایمنی بدن حیوان در مواجهه با عوامل خارجی نظیر لیپوپلی ساکارید باکتریایی به‌کار گرفت. اندازه گیری این فراسنجه ها در خلال اندوتوکسمی در حیوانات، دارای ارزش تشخیصی و پیش آگهی دهنده است. **هدف:** هدف از انجام این مطالعه تجربی، تبیین پاسخ فاز حاد به دنبال القای اندوتوکسمی توسط اشرشیاکولی سروتیپ O55:B5 در گوسفند دنبه دار ایرانی بود. همچنین بررسی همبستگی بین فراسنجه های پاسخ فاز حاد و الگوی تغییرات آنها در خلال اندوتوکسمی در گوسفند نیز از اهداف مطالعه حاضر بود. **روش کار:** تعداد ۵ رأس میش دنبه دار ایرانی به ظاهر سالم (با وزن بدنی ۵kg±۲۵) به طور تصادفی انتخاب شده و لیپوپلی ساکارید اشرشیاکولی سروتیپ O55:B5 به میزان ۲۰ µg/kg به صورت وریدی به هر حیوان تزریق شد. مایع درمانی به مدت ۱۲۰ دقیقه پس از تزریق لیپوپلی ساکارید انجام شد و ۱۸۰ دقیقه به طول انجامید. نمونه های خون از هر گوسفند، قبل، ۱، ۲، ۳، ۴، ۵، ۶ و ۲۴ ساعت پس از تجویز لیپوپلی ساکارید اخذ و سرم‌ها جداسازی شد. غلظت‌های سرمی هاپتوگلوبین، سرم آمیلوئیدآ، تومور نکروز فاکتور آلفا، اینترفرون گاما، گلوتاتیون پراکسیداز و سوپراکسید دیسموتاز مورد ارزیابی واقع شد. **نتایج:** پس از القای اندوتوکسمی، افزایش سریع و معنی دار هاپتوگلوبین، سرم آمیلوئید آ، تومور نکروز فاکتور آلفا و اینترفرون گاما مشاهده شد. غلظت سرمی گلوتاتیون پراکسیداز و سوپراکسید دیسموتاز پس از تزریق وریدی لیپوپلی ساکارید به طور معنی داری کاهش یافت. نتایج مطالعه تجربی حاضر نشان داد که هاپتوگلوبین، سرم آمیلوئیدآ، تومور نکروز فاکتور آلفا و اینترفرون گاما در تمام ساعات پس از القای اندوتوکسمی، همبستگی مثبت معنی داری با یکدیگر داشتند. این فراسنجه ها در تمام این ساعات به طور معنی داری با گلوتاتیون پراکسیداز و سوپراکسید دیسموتاز همبستگی منفی داشتند. **نتیجه‌گیری نهایی:** نتایج مطالعه تجربی حاضر می تواند شواهدی را از ارتباط بین پروتئین ها، سیتوکین ها و آنزیم های پاسخ فار حاد و تغییرات آنها در خلال اندوتوکسمی در گوسفند فراهم کند.

واژه‌های کلیدی: بیومارکرهای پاسخ فاز حاد، همبستگی، اندوتوکسمی، گوسفند دنبه دار ایرانی

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