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Annexin A1, A2 and cytokine levels during experimental sepsis in calves

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Keywords

annexin, calves, cytokines, *E. coli*

Abstract

Annexins are fundamentally related proteins that process a variety of physiologic and pathologic procedures, including suppression of inflammation. Ten Holstein-Frisian bull calves (10 ± 1 days old) weighting 50 ± 5 kg were chosen to induce the experimental septicaemia using O111:H8 strain of *E. coli*. Blood samples were collected to determine the plasma annexin A1 , annexin A2, TNF-α, IFN- γ, IL-8 and neutrophil count at 0, 24, 48, 72, 96 and 120 hours after induction of septicemia. Significant increased concentrations of serum annexin A1 and annexin A2 in circulating blood in response to experimental coliseptisemia were observed during experiment. Maximum levels of annexinA1 and A2 were recorded at 72h after challenge. A statistically significant increase in blood neutrophil count occurred from beginning of septicemia untill 24h after onset of septicemia. Annexin A2 and Annexin A1 had no significant correlation with neutrophil count. Serum cytokine concentrations reached their maximum level at 48h after challenge and then decreased to basal level before antibiotic therapy. This study showed that serum annexin concentrations, increasing during colisepticemia in calves, in association with cytokines could be a reliable marker to confirm the occurrence of anti-inflammatory response.

Abbreviations

CFU: Colony-Forming Unit
TNF-α: Tumor Necrosis Factor alpha
IFN: Interferon
IL-8: Interleukin 8
ELISA: Enzyme-Linked ImmunoSorbent Assay

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Introduction

The inflammatory reaction is a defensive process whereby the body is able to neutralize infections [1]. In the site of the systemic or local inflammation, polymorph nuclear leukocytes, lymphocytes, monocytes and endothelial cells activation lead to release of pro-inflammatory cytokines. Bacterial lipopolysaccharides activate the macrophages and cause release of TNF-α and IL-8 which can have an effect on heart, liver and other organs [2].

The pro-inflammatory phase is capable of inducing several endogenous anti-inflammatory mechanisms that lead to the resolution of inflammation phase. In response to injury, inflammatory cells such as neutrophil granulocytes secrete a number of cytokines into the bloodstream such as IL-1, IL-6, IL-8, and TNF-α [3,4]. Tumor necrosis factor α is a cytokine that has been associated with neutrophil extravasation and enrolment to inflammatory sites [5].

Annexins (also known as lipocortins) are a family of fundamentally related proteins which are classified by way of their ability to bind membrane phospholipids in a Ca^{2+}-dependent mode. One of the main roles of annexins is the regulation of a variety of physiologic and pathologic processes such as suppression of inflammation [3,4]. They are predominantly abundant in a number of cells of the host immune system. Annexin A1 can be transferred from cytoplasm to membrane after activation or after adherence to endothelial cells and be released from neutrophils [6,7,8] .

In normal conditions, cytoplasm of immune cells such as neutrophils, monocytes, and macrophages contains high levels of annexin A1. During inflammatory responses in calves, changes in neutrophils occur to a greater extent as compared to other cells. Following cell activation, neutrophils bind to endothelial-cell monolayers and annexin A1 is mobilized to the cell surface and secreted [8, 9]. Annexin A1 promotes neutrophil apoptosis and the apoptotic neutrophils are phagocytized by macrophages [8]. Annexin A1 is also released by apoptotic neutrophils during the process of inflammation and enhances the clearance of apoptotic cells by tissue macrophages that are able to mediate a rapid anti-inflammatory effect [8, 10].

Annexin A2 is involved in various biological functions such as fibrinolysis, angiogenesis, and cell migration, but the exact mechanism of its activity is not understood. In inflammatory dendritic cells, Annexin A2 maintains immunomodulatory activation of cytokines secretion, indicating an important role in normal situation and inflammatory diseases [10, 11].

Although extensive research has been carried out on cytokines and annexins during sepsis, there has been no reliable evidence that shows any correlation between these factors and time span of sepsis. On this basis, we conducted a study to determine the levels of annexins A1 and A2, IL-8, TNF-α, and IFN-γ in the peripheral blood of septic calves over a time span, and to investigate an association between serum Annexins A1 and A2 levels, blood neutrophil count, and serum cytokine levels.

Results

Evaluation of the results of the present study demonstrated that annexin A1 and annexin A2 were elevated in peripheral blood in response to experimental coliseptisemia and these changes over time were significant ($p < 0.05$). Maximum level of annexin A1 was recorded at 72h after challenge, and the serum levels of annexin A1 at 24, 48, and 72 h ($P = 0.039$, $P = 0.04$ and $P = 0.045$, respectively) were significantly higher than its level in 0 hour (Figure 1).

The serum levels of annexin A2 at 72 hours after colisepticemia increased significantly ($P = 0.017$) (Figure 2). Repeated measure ANOVA revealed significant changes in serum level of Annexin A2 over time after challenge ($p < 0.05$).

TNF-α, IL-8 and IFN-γ concentrations in this experiment reached to a maximum level at 48h after challenge. The serum concentrations of TNF-α, IL-8 and IFN-γ were significantly different during the experimental time points (Figures 3 and 4).

The neutrophil count showed significant changes during the study ($P = 0.004$) and its increase was statistically significant at 24 h after challenge as compared with its level at the beginning of septicemia ($P = 0.003$) (Figure 5). The white blood cell count decreased due to septicemia ($P = 0.0001$) (Figure 6).

Discussion

During the inflammation, bacterial immunogenic components stimulate production of pro-inflammatory and inflammatory factors and modify

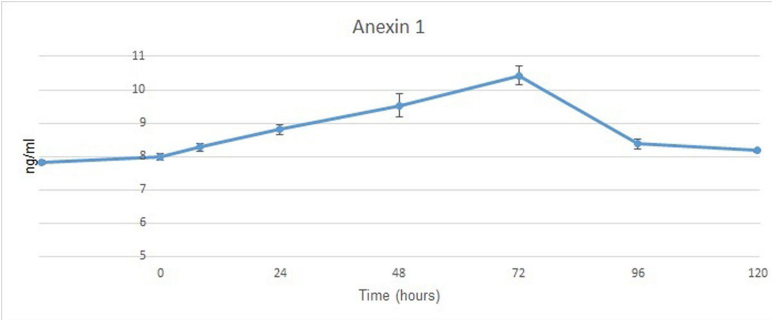

Figure 1
Variation of Annexin A1 level during experimental septicemia with *E.coli* (Mean ± SE).

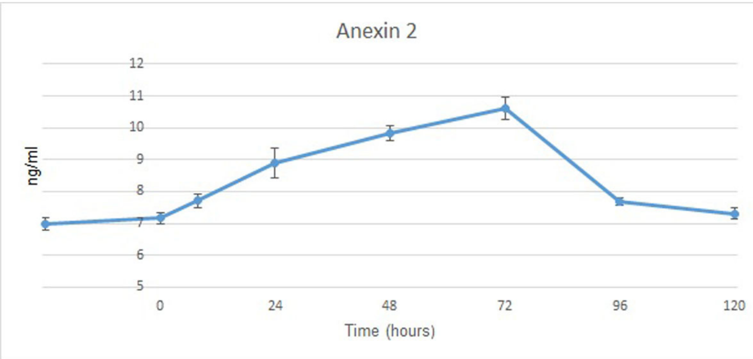

Figure 2
Variation of Annexin A2 level during experimental septicemia with *E.coli* [Mean ± SE].

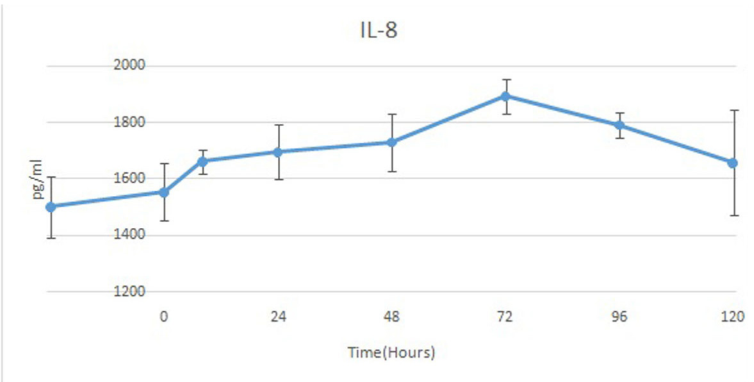

Figure 3
Variation of IL-8 level during experimental septicemia with *E.coli* (Mean ± SE).

blood cell numbers and amount of cytokines [2,8]. Neutrophils are critical components of the innate immune reaction and are the preliminary responders to infection. Twenty four hours after bacterial infection they increase in peripheral blood to limit the inflammation.

Activation of polymorphonuclear cells has an important role in sepsis including the release of pro-inflammatory cytokines such as TNF-α and IL-8, that in turn induce immune cell recruitment to the inflammation site [8]. The results of the present study showed that an elevated level of annexin A1 and annexin A2 in the peripheral blood of septic cases may play a part in an active anti-inflammatory function which subsequently contributes to the resolution of sepsis. The enhanced level of annexin A1 during experimental colisepticemia in calves in this study, with the highest recorded level in hour 72, is in agreement with the previous reports [6, 8]. Annexin A1 is an anti-inflammatory protein that plays a key role in innate immunity and modulates the activation of several types of cells such as neutrophils.

We found that in response to infection, the level of annexin A1 and A2 was increased in the peripheral blood after activation and proliferation of neutrophiles. The level of pro-inflammatory cytokines was significantly elevated in all cases with sepsis; and those levels correlated with the levels of AnnexinA1 and A2 in some time points [12].

Body's defense system suppresses mechanisms through the anti-inflammatory mediators

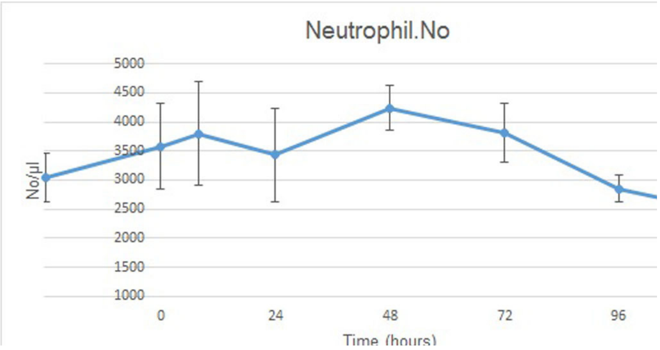

Figure 4
Variation of neutrophil number during experimental septicemia with *E. coli* [Mean ± SE].

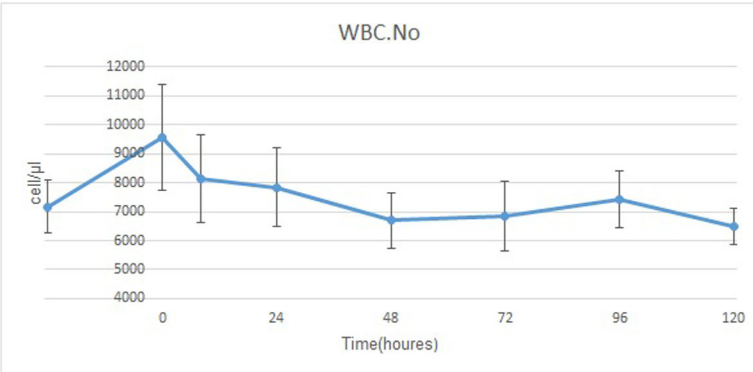

Figure 5
Variation of white blood cell number during experimental septicemia with *E. coli* [Mean ± SE].

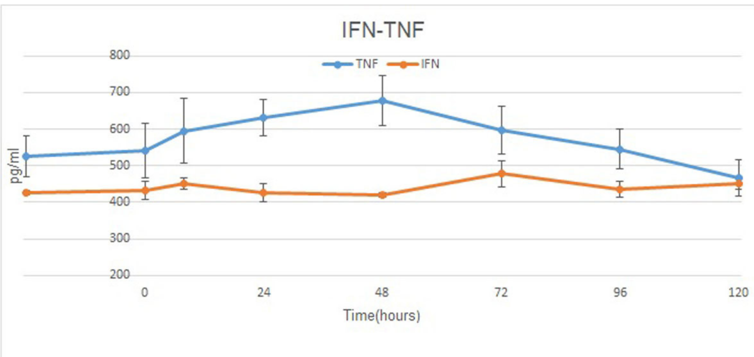

Figure 6
Variation of TNF-α and IFN-γ levels during experimental septicemia with *E. coli* [Mean ± SE].

such as TNF-α and IL-6 to resolve sepsis. Based on previous studies, serum cytokine disturbance patterns play a key prognostic role in septic shock cases and based on present results, serum annexin A1 reached its maximum level after 72 h compared to other cytokines[6].

In the present study, cytokines reached their peak level at 48h after challenge and decreased to basal level following antibiotic therapy. Annexin-A1 level had a mild increase after bacterial inoculation and treatment had no effect on its level. This delay in the enhancement of annexin levels may be due to the activation of neutrophiles that subsequently release annexins in blood circulation. On the other hand, annexin A1 has been shown to attenuate leukocyte recruitment in many experimental inflammatory models by inhibiting cell adhesion and migration [1, 18].

Based on Perretti and Gavins findings in 2003, cytokines such as tumor necrosis factor, interleukin-1, and interleukin-8 can also increase cellular and tissue annexin A1 expression [7]. It is clear that when neutrophils adhere to the endothelium and the amount of neutrophiles decrease in the circulation, annexin A1 is released from the neutrophil cytoplasm to the cell surface and thereupon the level of annexin is enhanced [7,14]. In fact, annexin A1 leads to the detachment of adherent leukocytes and indicates that inactivation of adherent cells may be controlled [7].

Based on previous studies, elevation of anti-inflammatory cytokines such as IL-10 and TNF-RI, due to sepsis can result into an enhanced risk of death. An elevation in concentration of annexins A1 and A2 after high level of cytokines found in this study can be an indication of the protective effects of annexins during sepsis[2,12]. However, it is unclear why the annexin A1 levels in the sepsis patients were not correlated in response to neutrophil counts. In previous reports in contrast to present results, annexin A1 levels in sepsis patients were not elevated in response to septicemia reaction. Further studies have to be done to investigate the role of circulating Annexin A1 & A2 in clinical applications among patients with sepsis[8].

Notwithstanding, there was no observed significant correlation between neutrophils count and annexin A changes but these findings suggest that increasing the annexins during the colisepticemia in calves after treatment can be a reliable marker with regard to cytokines, confirming the occurrence of anti-inflammatory response after activation of neutrophiles. It is hypothesized that the annexin proteins have anti inflammatory roles in the inflammation phase by decreasing the infectious cells and enhancing the immune defense factors. It is still a controversial issue as to whether or not the circulating level of anti-inflammatory mediators can be used as a prognostic factor to predict case survival.

Materials and Methods

Animals and experimental preparations

Ten Holstein-Frisian bull calves 10 ± 1 days of age with body weight of 50 ± 5 kg were selected for study. The calves were fed colostrum [10% BW] within six hours of birth. They were housed in individual stainless steel pens [1 m × 1.5 m × 1m] with a chaff coated floor and were fed twice daily with whole milk at the rate of 10% of their body weight per day divided into 2 feedings at 7:30 and 16:30 [15]. Water and starter provided ad libitum. The calves' vital signs (temperature, heart and respiratory rate) were checked before experiment [12,14].

The O111:H8 strain of *Escherichia coli* was chosen for inducing colisepticemia. This strain is commonly used in experimental studies since it is rapidly phagocytized, and produces a robust oxidative burst [16,17]. To induce experimental septicemia, a catheter was inserted in the jugular vein and an *E coli* suspension [1.5×10^9 CFU] in 5 ml isotonic saline was inoculated as a bolus.

Biochemical and hematological analysis

After observation of the septisemia symptoms based on accepted criteria including altered appetite and behavior, shock signs, standing ability, and suckling reflex and hematology confirmation, blood samples were collected into 6-mL tubes containing EDTA for determination of plasma annexin A1, annexine A2, TNF-α, IFN-gamma, IL-8 and blood cell count at 0 [inoculation time], 24, 48, 72, 96 and 120 hours after challenge. Four ml of peripheral blood was collected into a sterile syringe after monitoring the calves and observing the septic shock symptoms and injected to a Diphasic media and incubated at 37°C for 24 h to confirm septicemia in calves. Then pure culture and serotyping was performed to detect the isolated bacteria and to confirm the *E. coli* strain O111:H8. The measurement of serum levels of annexin A1 and annexin A2 were carried out by ELISA (Cusabio, Australia). The serum levels of TNF-α, IFN γ and IL-8 were determined by related ELISA kits (AbD Serotec®, A Bio-Rad Company, Kidlington, Uk).

For ethical reasons, all calves were treated with a suitable antibiotic which had been selected by antibiogram. Treatment with antibiotic started 36h after bacterial administration with Ceftazidime (ZACZIDIM®, DAANA Pharma Company, Tehran, Iran) at a dose of 10 mg/kg IV TID for 3 consecutive days.

Statistical Analysis

The data were analyzed with repeated measures ANOVA using SPSS version 13.0 software and significance level was considered as p less than 0.05. The correlation between annexin A1 and A2 and cytokines concentration and neutrophile counts was studied using Pearson's correlation coefficient.

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Author Contributions

Desinged the study and conducted the systematic literature review: Z.E., M.R.M.D. and M.M.D. Analyzed the data and drafted the manuscript: Z.E. Conducted the experiments and participated in *in-vivo* studies: Z.E. M.R.M.D. and M.H.S.

Conflict of Interest

The authors declare that they have no competing interests.

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