MAE Vet Fak Derg, 8 (2): 130-135, 2023
DOI: 10.24880/maeuvfd.1314627

Research Article

Decreased gene expression of RIPK1 and RIPK3, necroptosis players, in calves with sepsis

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Key Words:
calves
necroptosis
RIPK1
RIPK3

Received : 16.06.2023
Accepted : 02.08.2023
Published Online : 31.08.2023
Article Code : 1314627

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This work was based on a Master of Science Thesis and supported by Burdur Mehmet Akif Ersoy University Scientific Research Projects Unit Under Project number 0522-YL-18.

ABSTRACT

As the world's population increases, so does the need for livestock-based nutrition. In addition, the livestock sector becomes more important as it contributes to the economy. However, sepsis has high morbidity and mortality rate in newborn calves and can cause severe economic losses. Therefore, new biomarkers to distinguish sepsis from other diseases are urgently needed in veterinary medicine. This study, for the first time, examined the gene expression levels of members of the necroptosis pathway, such as receptor-interacting serine/threonine protein kinase 1 (RIPK1), RIPK-3, and one of the NF-κB activating proteins, RIPK-2, in septic calves. We examined the mRNA levels of RIPK1, RIPK3, and RIPK2 using qPCR in 10 healthy Holstein calves and 20 Holstein calves with sepsis due to suffering from enteritis infection between 1-20 days of age. The hematologic parameters, including leukocytes, erythrocytes, hemoglobin, hematocrit, and platelets, were also evaluated in the calves included in this study. The results showed that calves with sepsis had prominently lower mRNA levels of RIPK1 and RIPK3 than those in healthy calves. Besides, RIPK2 mRNA expression was absent in healthy calves and calves sepsis. In veterinary medicine, decreased RIPK1 and RIPK3 mRNA levels might be biomarkers to diagnose sepsis in calves.

INTRODUCTION

The livestock sector provides animal protein to society and raw materials to the dairy, textile, leather, cosmetics, and pharmaceutical industries. Therefore, livestock is essential for all countries, as it contributes significantly to the economy. Sepsis following diarrhea can cause severe economic losses in calves (Fecteau et al., 1997; Guzelbektes et al., 2022). Sepsis is a complex overreaction described as the systemic hyperinflammatory immune response associated with a proven or suspected infection in animals and humans (Jarczak et al., 2021). In humans, it can cause multi-organ failure and lead to death in up to fifty percent of cases (Nedeva et al., 2019). Organ failure occurs after strong immune system activation by initiating the inflammatory response to eliminate pathogens by binding pathogen-associated molecular patterns (PAMPs) to their recognition receptors. Damage-associated molecular patterns (DAMPs) are then released by dying host cells to trigger the elevation of inflammatory cytokine levels that amplify cytokine storms. Organ failure and excessive organ infiltration of leukocytes are hallmarks of severe sepsis caused by cytokines, PAMPs, and DAMPs that can be released into the circulation from dying cells (Nedeva et al., 2019).

Necroptosis, a distinctive form of regulated non-apoptotic cell death, is triggered by death receptors such as tumor necrosis factor receptor 1 (TNFR1), TNFR2, and Fas. Receptor-interacting serine/threonine protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL) which is a direct executor, are central to necroptosis. The translocation of MLKL to the cell membrane results in the rupture of the membrane and the release of intracellular contents and organelle swelling. In particular cell types and conditions, some PAMPs and DAMPs are associated with necroptosis induction (Kaczmarek et al., 2013). Studies revealed that the mortality of mice with TNF-induced systemic inflammatory response syndrome (SIRS) was driven by RIPK1-RIPK3-mediated necroptotic cell death (Duprez et al., 2011). In mice subjected to polymicrobial sepsis, RIPK3 promoted sepsis to induce acute kidney injury (Duprez et al., 2011). Moreover, necroptosis caused hepatic damage in piglets with lipopolysaccharide-induced sepsis (Xu et al., 2021). RIPK1 and RIPK3 activities have been linked to necroptosis. At the same time, RIPK2 plays essential roles in innate immunity and in the activation of the NF-kb, which has been implicated in the pathogenesis of organ injury and lethality during sepsis (Li et al., 2009; Bullock et al., 2015).

In human patients with sepsis, procalcitonin, and presepsin

To cite this article:
Doğan A, Baykan Y, Akcora Yıldız D. Decreased gene expression of RIPK1 and RIPK3, necroptosis players, in calves with sepsis. MAE Vet Fak Derg. 2023; 8 (2) :130-135.

are currently used as biomarkers to detect sepsis, and new biomarkers have been investigated for precise diagnosis of sepsis. In addition, most acute phase proteins, including C-reactive protein, are used as biomarkers in animals with suspected sepsis. However, the amount of acute-phase proteins can also be elevated in other inflammatory diseases. In addition, sepsis and sepsis-related mortality rates in calves are poorly explored, but it is estimated that up to 30% of calves with neonatal diarrhea or illness are septic (Lofstedt et al., 1999; Fecteau et al., 2009). Thus, there is a need for new biomarker studies in terms of early diagnosis and prognostic follow-up of sepsis. Therefore, we evaluated the mRNA expression levels of RIPK1, RIPK3, and RIPK2 in calves with sepsis.

MATERIALS and METHODS

Animals

This study included10 healthy (control group) and 20 sepsis (experimental group) Holstein calves aged 1-20 days. In addition, routine clinical examinations of the diarrheal calves brought to the large cattle clinic were performed, and calves with sepsis, according to clinical and laboratory findings, were included in this study. The ethics committee approval required was obtained by Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee on 16/12/2018. The presence of at least two or more recorded SIRS signs proven infection or infection with reduced or absent sucking reflex, lack of interest in the surroundings, inability to stand up without support, or lateral recumbency was defined as sepsis (Fecteau et al., 1997; Fecteau et al., 2009; Singer et al., 2009; Aygun and Yildiz, 2018).

Sample collection and analysis Blood samples for hemogram analyses from the experimental and control groups were taken from the *jugular vein*before treatment during routine blood tests. Tubes with ethylenediaminetetraacetic acid (K_3EDTA) were used for hemogram measurement, tubes with activator gel for serum and whole blood was used for RNA extraction and qPCR. A hemogram analysis was performed within 15-30 minutes. In addition, parameters such as leukocytes (WBC), granulocytes, erythrocytes (RBC), hematocrit (HCT), and platelets (PLT) from the venous blood sample with K_3EDTA were measured using the Abacus Junior Vet (Diatron MI Ltd. Hungary).

RNA extraction

Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the total RNAs from blood samples according to the manufacturer's instructions to identify RIPK1, RIPK2, and RIPK3 gene expression levels. RNAse-free water was used for the dissolution of RNAs. The integrity of RNAs was demonstrated on %1 agarose gel, and the concentration of these RNAs was analyzed using the BioTek spectrophotometer (Epoch, BioTek, Germany).

Real-time quantitative Polymerase Chain Reaction (qPCR)

The mRNA expression levels of RIPK1, RIPK2, and RIPK3 in samples of interest were quantified using Real-time quantitative PCR (qPCR). cDNA was then synthesized from RNA using the iScript Reverse Transcription kit (Biorad, Hercules, CA, USA) following the protocol provided by the manufacturer. qPCR analysis of RIPK1, RIPK2, and RIPK3 mRNA expressions in healthy and septic Holstein calf blood samples was performed using the SYBR Green PCR Master Mix on LightCycler 480 Instrument II (Roche, Basel, Switzerland). GAPDH was used to normalize RIPK1, RIPK2, and RIPK3 mRNA levels. Table 1 shows the sequences of primers used in the present study. The amplification parameters for qPCR were as 95°C for 10 min, followed by 50 cycles of 95°C for 10 sec, 65°C for 30 sec, and 72°C for 1 sec.

Statistical analysis

GraphPad Prism 6 for Windows® package program was used to analyze the obtained data statistically. During the statistical evaluation of the data, an unpaired t-test was used to determine the significant differences between the two groups in terms of a parameter. $p < 0.05$ values were considered statistically significant.

RESULTS

Clinical assesment and hemogram findings

Decreased sucking reflex, depression, lack of interest in consciousness, weakness in standing up, hypothermia or hyperthermia, prolongation of capillary filling time, abdominal respiration, hyperemia or pallor of mucous membranes were observed in calves with sepsis. The hematological and clinical parameters, including body temperature, respiration, and pulse rate of the calves of the experimental and control groups and are presented in Table 2. In the hemogram analysis, leukocyte (WBC) ($p < 0.01$) and granulocyte ($p \leq 0.01$) counts were significantly increased in calves with sepsis compared to healthy calves. However, RBC, HCT and PLT levels were not found to be statistically significant. In addition ieukopenia was detected

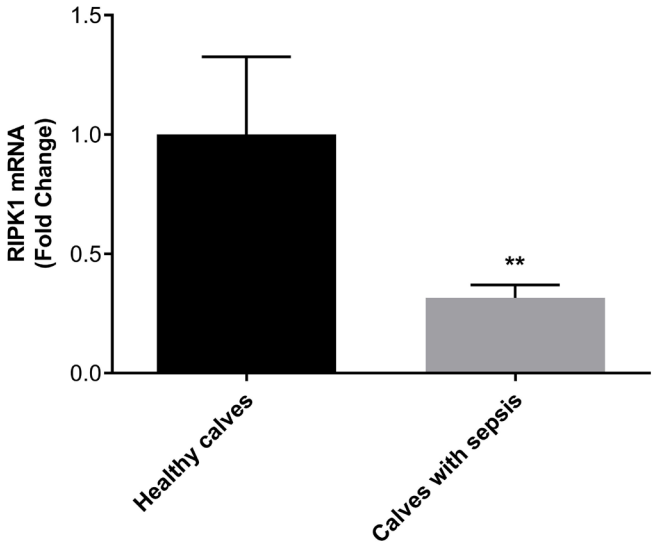

Figure 1. Decreased RIPK1 mRNA expression in calves with sepsis. The mRNA expression levels RIPK1 were examined in blood samples of health calves (n=10), and in blood samples of age-matched calves with sepsis (n=20) using qPCR. GAPDH was used to normalize RIPK1 mRNA levels. Values indicate mean ± SEM. Student unpaired t-test **$p < 0.01$ ($p=0.0054$).

Table 1. Sequences and annealing temperature (ºC) of primers used in this study

Gene Name	Primer Sequence (5'-3')	Annealing temperature (ºC)
RIPK1-F	5' ATTCCATTCACCTCCTTGCC 3'	57
RIPK1-R	5' GAACTCATTCCACCAATCTCCA 3'	58
RIPK2-F	5' CAGTGAATCACAGTCGGAACAG 3'	60
RIPK2-R	5' AAGCACAAGTATTTCTGGGTAAGG 3'	59
RIPK3-F	5' CCGAATAAACACCAAGAAAGCC 3'	58
RIPK3-R	5' TCAGAAAGGAACTCCTTCACCA 3'	58
GAPDH-F	5' GGTCACCAGGGCTGCTTTTA 3'	59
GAPDH-R	5' AGGATCTCGCTCCTGGAAGA 3'	59

Table 2. Hemogram and clinical findings of sepsis and control groups (Mean±SEM)

Parameters	Control (n:10)	Sepsis (n:20)	p value
WBC$^{(10^9/l)}$	8.18±0.49	21.2±3.79	0.003
GRA$^{(10^9/L)}$	3.65±0.49	13.5±3.34	0.008
RBC$^{(10^12/l)}$	8.74±0.73	10.2±2.38	0.552
HCT (%)	27.9±2.21	29.3±1.84	0.626
PLT$^{(10^9/l)}$	572±121	670±76.2	0.504
Temperature (°C)	38.9±0.09	37.3±0.29	0.00
Breath (min)	36.3±0.73	45.6±4.08	0.04
Pulse (min)	118±2.72	105±6.20	0.06
Gender	6 male, 4 female	7 male, 13 female	

WBC = white blood cell, GRA = granulocytes. RBC = red blood cells, HCT = haematocrit, PLT = platelets.

in 2 calves, and leukocytosis was detected in 18 calves with sepsis.

Results of the mRNA expression levels of RIPK1, RIPK2, and RIPK3RIPK1 mRNA expression levels were statistically decreased approximately threefold in calves with sepsis compared to healthy calves based on qPCR results (Figure 1).Similarly, RIPK3 mRNA levels were lower in calves with sepsis compared to their controls (Figure 2).

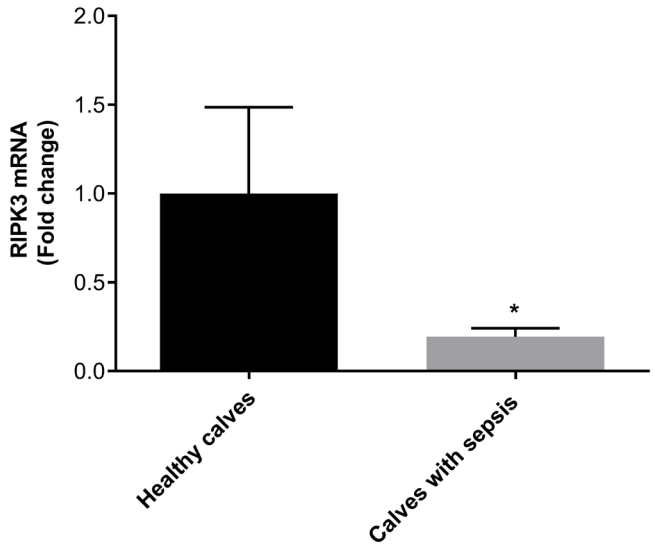

Figure 2. Lower RIPK3 mRNA expression in calves with sepsis. RIPK3 expression was evaluated by qPCR in blood samples of health calves (n=10), and in blood samples of age-matched calves with sepsis (n=20). Values indicate mean ± SEM. Student unpaired t-test **p < 0.05 (p=0.0267).

However, RIPK2 expression could not be determined in both the healthy calves and the calves with sepsis, probably due to its low expression (graph not provided).

DISCUSSION

Neonatal sepsis remains one of the most significant health problems in cattle breeding because of its high morbidity and mortality rate. Despite the development of sepsis treatment options, new biomarkers are needed for early diagnosis and prognostic follow-up of sepsis. In this study, the mRNA expression levels of RIPK1 and RIPK3, crucial players in the necroptosis cell death pathway, were investigated for the first time. We found that the gene expression levels of RIPK1 and RIPK3 were significantly decreased in the calves with sepsis compared to those in healthy calves. Moreover, pro-inflammatory inducer RIPK2 expression was not detected in calves by qPCR.

Sepsis has a complex and multifactorial pathophysiology. In general, the pro-inflammatory process is stimulated by the pathogen, and it accompanies the elimination of the pathogen, but the host activates the anti-inflammatory process to repair the tissues. The imbalance of these mechanisms can cause excessive tissue damage (pro-inflammatory) or immunosuppression in the body and predispose it to secondary infections

(anti-inflammatory). The organ's response to these events depends on the features of the defense of the host (morbidity and immunosuppression) and the pathogen (virulence and amount of organism) (Angus and Van der Poll, 2013). Numerous defense mechanisms are emerging for pathogen control and elimination, including inflammatory cytokine, interferon, and chemokine production, adaptive immune response, and activation of cell death pathways, including necroptosis (Fecteau et al., 1997; Guzelbektes al., 2022). Necroptosis is a genetically regulated necrotic cell death pathway that is thought to kill pathogen-infected cells and/or damaged cells in inflammatory pathologies. Necroptosis is mediated by TNFR and Fas, or pathogen recognition receptors, including tTLRs and Z-DNA binding protein 1 (Qu et al., 2022). RIPK1, RIPK3 and MLKL have been identified as critical molecular components of necroptosis (He et al., 2000; Cho et al., 2009; Holler et al., 2009; Zhang et al., 2009). The binding of TNF to TNFR leads to the recruitment and phosphorylation of RIPK1, the adaptor protein TRADD (death domain-associated TNFRSF1A). RIPK1 then activates RIPK3, which in turn phosphorylates MLKL kinase. After phosphorylation, MLKL undergoes significant conformational changes that allow its insertion into the cell membrane and induce membrane permeability by creating pores.RIPK3 has been identified as the essential molecule for TNF-induced necroptosis (Upton et al., 2010; Moujalled et al., 2013), while RIPK1 has an essential role in mediating both TNF-dependent nuclear factor κB (NFκB) activation and apoptosis (Ofengeim and Yuan, 2013). In the present study, RIPK1 and RIPK3 mRNA levels were lower in the calves with sepsis (experimental group) than in the healthy calves. Duprez et al.'s (2011) study emphasized that inhibition of RIPK1 and RIPK3 is essential in preventing SIRS caused by TNF. Studies showed that necroptosis increases in direct proportion to the severity of sepsis when severe sepsis and septic shock were compared with sepsis in humans (Duprez et al., 2011; Wang et al., 2012). Evidence supporting our study was the relationship between inflammation and necroptosis in a mouse model of TNF-induced SIRS (Kaiser et al., 2013). RIPK3 deficiency has been reported to protect against lethal SIRS and reduce circulating DAMPs (Kaiser et al., 2013). It is thought that the calves with sepsis taking place in our study may be in the initial stage of sepsis, and the RIPK3 level may decrease temporarily, but multi-organ dysfunction findings will occur due to the development of necroptosis and apoptosis in the later stages. A recent study revealed that RIPK3 cooperates with the lethal gesdarmin D (GSDMD), which is involved in pyroptosis, to increase tissue damage in polymicrobial sepsis (Chen et al., 2020). Briefly, this study reported that both necroptosis and pyroptosis are associated with caecal ligation and perforation (CLP)-induced sepsis and multi-organ dysfunction, and the use of agents that inhibit the activity of these molecules may protect against septic shock and multi-organ damage (Chen et al., 2020).

Han et al. (2019) showed that necroptosis is involved in the pathogenesis of acute-on-chronic hepatitis B liver failure (ACHBLF). For that, they measured the mRNA levels of RIPK3 using qPCR and the protein levels of MLKL by ELISA in the peripheral blood of healthy controls and patients with ACHBLF and chronic hepatitis B. They found that RIPK3 mRNA levels were significantly higher in patients with ACHBLF than those with chronic hepatitis B or healthy controls, positively correlated with serum MLKL. Moreover, the mRNA expression of MLKL, RIPK3, and Beclin-1 was assessed in 45 primary immune thrombocytopenia (ITP) patients' peripheral blood and 20 healthy controls' peripheral blood. The parameters of clinic and laboratory and patients' response to steroid therapy were evaluated with mRNA expression of MLKL, RIPK3, and Beclin-1. It was found that there is a crosstalk between increased mRNA expression of RIPK3 and MLKL and autophagy-related protein Beclin-1 in primary immune thrombocytopenia (Kamal et al., 2022). Moreover, the production of RIPK3 mRNA and protein expression showed correlation with the presence of necroptosis signaling in melanoma cell lines (Geserick et al., 2015). All these studies indicate the correlation between mRNA and protein levels of RIPK3.

RIPK2 plays an essential role in forming the immune response by intracellular nucleotide binding and oligomerization domain (NOD)-mediated NF-kB activation and cytokine production (Nachbur et al., 2015; He et al., 2017). NOD receptors recognize antigens containing bacterial peptidoglycans and initiate immune responses by activating NF-κB and MAP kinases, triggering the production of pro-inflammatory cytokines (Jun et al., 2013). No study has been found investigating the potential role of RIPK2 in the occurrence of bacterial sepsis. In our study, the mRNA expression level of RIPK2, which has a role in forming the immune response against bacterial infection, could not be determined in most healthy and septic calves. This is thought to be due to the late arrival of RIPK2 in the cycle and the differences between individuals.

CONCLUSION

In summary, RIPK1 and RIPK3 may contribute to the early diagnosis and understanding of the therapeutic effect of neonatal sepsis in calves. The combined detection of RIPK3 with proven biomarkers such as c-reactive protein and procalcitonin may be more effective than individual ones in diagnosing neonatal sepsis. Moreover, studies are required to compare the changes in RIPK1 and RIPK3 expressions in the treatment processes of patient groups (sepsis, severe sepsis, and septic shock) with healthy calves. In addition, investigating the role of RIPK1 and RIPK3 in the treatment process in calves will contribute to understanding their potential to be used as mortality and prognostic indicators and the mechanisms that contribute to increasing treatment success. This study's results suggest that lower RIPK1 and RIPK3 mRNA levels may be associated with the beginning of sepsis. Furthermore, RIPK2 may not contribute to the induction of innate immunity in calves with sepsis.

DECLARATIONS

Ethics Approval

The present study was approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Protocol 16-12-2018).

Conflict of Interest

The authors have no conflicts of interest.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: AD, DAY

Data collection and analysis: AD, YB, DAY

Drafting of the manuscript: AD, YB, DAY

Critical review: AD, YB, DAY

Data Availability

Data were available on request from correspondence author.

Acknowledgements

We would like to thank Assoc. Prof. Ramazan Yıldız for kindly providing the clinical samples and clinical data used in the present study.

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