Dynamic of Cytokine Gene Transcription (TNF-α, IL-1β, IL-6, IL-8) in Surgically Treated Colic Horses by Use of Real-Time PCR (RT-PCR)

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ABSTRACT

Horses are highly sensitive to endotoxemia and its complications. During systemic inflammatory response syndromes such as seen in colic, acute phase proteins and cytokines enter into the circulation. In this study, gene expression of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8) were quantified using real-time PCR (RT-PCR) in 15 adult horses undergoing colic surgery at different points of time (0, 6, 12, 24, 48 hours), before and after surgical intervention. Gene expression of TNF-α was down-regulated while gene expression of IL-1β, IL-6 and IL-8 were up-regulated in the first 12 hours. This study shows that RT-PCR is reliable and sensitive method for detection of the changes in cytokines serum levels in horses with colic. This study demonstrated the presence of inflammatory processes mediated by cytokines upon admission of horses for medical treatment before surgical intervention.

Keywords: Horse; Colic Endotoxemia; Cytokines; RT-PCR; TNF-α.

INTRODUCTION

Colic is one of the most common pathologies in equine medicine requiring immediate medical and in some cases surgical intervention (1, 2). During a colic event the horse maybe suffering from a combination of intestinal hypoperfusion, stasis, ischemia and increased permeability, resulting in transmural and transvascualar bacterial and their associated endotoxin migration (4). Colic and its associated endotoxemia has been found to induce release of acute phase proteins and cytokines resulting in cardiovascular and gastrointestinal dysfunction complement activation, organ failure and death (1, 4, 5, 6).

Real-time PCR (RT-PCR) methodology is increasingly used for determining cytokine production in humans and animals (7, 8) due to the accuracy and reliability of the method (7, 9). Results from human and laboratory rodent studies suggest that cytokine expression levels along with clinical signs can improve diagnosis, treatment and prognosis of patients with endotoxemia and sepsis (10, 11, 12). This method has been described in septic foals and in horses with infiltrative inflammatory bowel disease and horses with herpes virus infection (13, 14, 15, 16, 17). Expression of cytokine mRNA in neonatal foals has been used as a surrogate for sepsis and bacterial virulence (13). To the best of knowledge of the authors, measurement of cytokine gene transcription has not been reported in adult horses undergoing colic surgery. We hypothesized that gene expression of selected blood biological markers (i.e. TNF-α, IL-1β, IL-6, IL-8) can be detected by using RT-PCR technique.

MATERIALS AND METHODS

Animals

Fifteen adult horses between the ages of 2-23 years of age were included in this study. All the horses were presented to the Koret School of Veterinary Medicine – Veterinary Teaching Hospital (KSVM-VTH) between the years 2009-2011 with abdominal colic and were treated surgically. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem.

Study design

Data retrieved from files which included physical examination parameters, routine blood analysis [complete blood count (CBC), packed cell volume (PCV), total solids (TS), urea and lactate] upon admission and during the following 48 hours of the hospitalization period. Five blood samples were collected from the jugular vein in Tempus TM Spin RNA isolation blood tubes (Applied Biosystems, Foster City, California) during the hospitalization period; at induction of general anesthesia before surgery (time 0) and after 6, 12, 24 and 48 hours. Blood samples were kept at -20˚C until analysis.

The decision to perform surgical intervention was carried out by an expert board certified equine surgeon based on clinical signs and clinicopathological findings. Severity of the disease was graded as mild, moderate or severe based on intraoperative findings (perfusion state of intestine, motility, and invasiveness of surgery procedure)

Anesthetic protocol

The anesthesia protocol was similar in all horses and included premedication with xylazine (AnaSed, Lloyd, Shenandoah, Iowa, USA) 0.8 mg/kg intravenously, induction with ketamine (Clorketam, Vetoquinol, Paris, France) 2.2mg/kg and diazepam (Assival, Teva pharmaceuticals, Petach-Tikva, Israel) 0.05 mg/kg intravenously, and maintenance with isoflurane inhalant anesthesia (Nicholas Piramal, Andhra Pradesh, India) delivered in 100% oxygen. All horses were mechanically ventilated during the anesthetic period. Monitoring during anesthesia included; respiratory rate, pulse rate and quality, direct blood pressure, ECG and end tidal CO2 (ETCO2). Dobutamine (Taro Pharmaceutical Industries Ltd., Haifa, Israel) was administered in constant rate infusion in order to maintain mean arterial blood pressure above 80 mmHg. Lidocaine (Esracaine, Rafa, Jerusalem, Israel) was administrated to 8/15 (53.3%) of the horses as bolus of 1.8 mg/kg immediately after induction of anesthesia followed by constant rate infusion of 50 µg/kg/min for the next 24 hours based on the preference of the anesthesiologist.

PCR (RT-PCR) for relative measurement of cytokine-specific mRNAs

Since a detection system that quantifies horse cytokines is not available, and as most cytokines are transcriptionally regulated, cytokine induction and quantification was assessed through cytokine messenger RNA (mRNA) transcripts levels. Unrelated studies, comparing between the rise of mRNA transcripts and the (ELISA) quantification of cytokines have shown that these correlate, and that the assay is reproducible (18, 19, 20).

cDNA extraction

Extraction of RNA was performed according to the protocol of Applied Biosystems Tempus TM Spin RNA Isolation by Nanodrop (ND 1000), and storage at -80˚C up to use. Dilution of samples was performed in order to obtain similar concentrations of RNA in all samples (400ηg/µl). Ten µl was taken from each sample and diluted with 10 µl of standard reagents (buffer 4 µl, H_2O 1 µl, DNTP 2 µl, Enhancer1 µl, enzyms1 µl and primer 1 µl) (verso cDNA kit, Thermo Fisher Scientific). RT-PCR reaction to product cDNA was carried out with PTC-200 DNA engine according to protocol of P7S1; first cycle for 30 minutes in 42˚C and second cycle for 2 minutes at 95˚C. Final volume of 20 µl of cDNA was kept at -20˚C. To normalize differences in quantity, stability and expression between the samples, the reference, housekeeping gene GAPHD used, which is in wide use for RT-PCR in horses. Primers for RT-PCR readings were stabilized to between 100-200 base ranges. Each gene primer was read in both directions in order to maximize sensitivity of the test.

RT-PCR reaction

The amount of expression of cytokines was determined by the RT-PCR method used by Absolute Blue SYBR Green Rox Mixes (Thermo scientific) according to the manufactor instructions. Mixture for PCR included SYBR Green Mix 5 µl 2.5 µl cDNA (constant concentration of 1.4ηg/µl), 0.15 µl forward primer, 0.15 µl reverse primer and 2.2 µl of water

(final concentration 300nM). Each well contained 10 µl. Each plate had two rows for the same sample in order to double-check results.

The reaction was carried out using StepOne Real Time PCR (Applied Biosystems, USA), one cycle 95˚C for 3 minutes, 35 cycles 95˚C 30 seconds, 55˚C for 30 seconds and 72˚C for 50 seconds. Finally, 45 cycles at 72˚C for 5 seconds with increase of temperature every two cycles by half a degree centigrade for determination of temperature for increasing given gene.

Analysis of results

The amplification efficiency of each cytokine was reported compared to GADPH mRNA expression (internal control), through evaluating and analyzing comparative threshold method (ΔCt) variation (final amount of cDNA template = 25ηg/well). Relative quantification (RQ) was obtained using the 2-ΔΔCt method (21) by adjusting the mRNA cytokine expression to the expression of β-actin mRNA and considering the adjusted expression in the control group as reference (relative quantification RQ = 1). Data were analyzed by the Applied Biosystems StepOneTM software v2.0 and expressed as RQ. Descriptive statistics (mean ± standard deviation of mean) was carried out to describe RQ in both in vivo and in vitro experiments.

Statistics

All parameters were normally distributed and reported as mean ± standard deviation (SD), were compared between the expressions of different cytokines by Student's *t*-test. Repeated measures ANOVA were used to assess changes in continuous variables over time. For determination of influence of onset of clinical signs to surgery, Fisher's Exact Test was used. For determination if the severity of disease has influence of cytokine expression, Kruskal-Wallis Test was used. Spearman's rank correlations were used to assess the correlation between continuous variables of the cytokine level in the different sampling times. For all test applied $P<0.05$ was considered statistically significant. All calculation was performed using statistical software. (SPSS 17.0 for Microsoft Windows, SPSS Inc., Chicago, IL, USA.)

RESULTS

Fifteen horses, 2-23 years of age participated in the study, with mean age of 11 years (SD ± 6.81 years). Mean lag time from the onset of clinical signs to the surgery was 13.4 hours (SD ± 16.99 hours). Data on admission for all the horses are presented in Table 1. There was no correlation between all the measured cytokine expression (TNF-α p=0.949, IL-1β p =0.820, IL-6 p=0.102, IL-8 p=0.151) and lag time from the onset of clinical signs to surgery. There was no correlation between the severity of disease and cytokine expression at the first sampling time during induction before surgery (TNF-α p=0.322, IL-1 β p=0.747, IL-6 p=0.396, IL-8 p=0.946). Lactate levels upon admission was not statistically significant (p= 0.903) different between mild to moderate and severe classified severity groups (26.80±13.72, 30.45±17.00 and 39.25±25.85, respectively).

Mean heart rate upon admission was 54.1 beats per minute (SD ± 11.5 BPM) and mean respiratory rate of 22.7 breaths per minute, (SD ± 10.7 breaths per minute). There was no correlation between clinical signs (heart and respiratory rate, temperature, and mucosal membrane color), and cytokine expression at admission and during hospitalization period.

In 8 of 15 (53%) horses, increased expression of IL-1β was seen at induction before surgery (time 0) comparing with reference gene GAPDH (RQ≥1). In 11 of 15 (73%) and in 13 of 15 (87%) horses IL-6 and IL-8 were increased at the first sampling time at induction before surgery All three interleukins reached peak levels 6 hours post induction and then gradually decreased over time (Figure 1). In contrast, TNF-α levels in 13/15 (87%) horses, were lower than GAPDH (RQ≥1) at anesthetic induction, reaching minimal level after 12 hours and then gradually increasing over time but staying below the reference range for all measurements (Figure 1). There was no difference in cytokine expression profile between horses treated with lidocaine and untreated horses for all measured points during 48 hours of hospitalization period.

DISCUSSION

The pathophysiology of colic is characterized by disturbances in gastrointestinal blood flow, leading to an increase in intestinal permeability (22). This may result in leakage of endotoxins into the blood stream and consequently may lead to systemic endotoxemia, systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS) and death (23). On the other hand, anti-inflammatory cytokines that are released later in the course

Table 1: Age, basic physical parameters, lactate level, diagnosis, severity of the colic and cytokine level in 15 horses surgically treated for colic upon admission.

Horse	Age years	HR[1]	RR[2]	MMC[3]	Time*	Operative diagnosis	Lactate	Severity**	TNFα[4]	IL-1β[5]	IL-6[6]	IL-8[7]	Lidocaine***
1	4	60	40	hyperemic	12 hrs	Large intestine impaction	25.2 mg/dl	moderate	0.376	3.5	0.18	6.59	yes
2	2		40	Pale dry	24 hrs	Cecum torsion	30.6 mg/dl	moderate	0.172	0.236	0.48	0.461	yes
3	10	32	16	pink	6 hrs	Large colon torsion	32.4 mg/dl	mild	2.02	3.62	4.32	3.62	yes
4	2	48	16		48 hrs	Large colon impaction	13 mg/dl	mild	0.53	0.39	1.96	4.37	yes
5	4	64		dry	6 hrs	Pelvic flexture impaction	4.5 mg/dl	moderate	1.08	0.88	4.82	3.42	yes
6	17	44		Pale dry	4 hrs	Large colon volvulus	30.6 mg/dl	severe	0.402	0.752	2.63	2.99	yes
7	11	72		purple	6 hrs	Pelvic flexture sand impaction	20 mg/dl	severe	0.349	0.405	42.48	6.33	yes
8	18	56	32	pink	6 hrs	Epiploic entrapment	29 mg/dl	severe	0.694	1.96	2.78	3.57	yes
9	9	72	12	pale	8 hrs	Large intestine torsion	77.7 mg/dl	severe	0.199	2.828	2.2	3.377	no
10	23				8 hrs	Small colon impaction	54 mg/dl	moderate	0.172	5.39	4.64	5.02	no
11	17	40	30	pale	12 hrs	Nephrospl. entrapment	NA	severe	0.305	3.966	0.33	0.85	no
12	3	40	20	Yellow pale	12 hrs	Diaphragm. hernia	7.2 mg/dl	mild	0.459	0.664	0.829	1.575	no
13	20	60		hyperemic	12 hrs	Nephrospl. entrapment	41.4 mg/dl	mild	0.838	0.852	1.509	2.96	no
14	10	60	16	Pale dry	6 hrs	Nephrospl. entrapment	39 mg/dl	mild	0.315	3.5	1.56	8.19	no
15	12	56		Pale dry	12 hrs	Nephrospl.entrapment	28.8 mg/dl	mild	0.528	1.616	2.083	2.3	no

1. heart rate per minute; 2. respiratory rate per minute; 3. mucous membrane color; * lag time from the onset of clinical signs to admission to the hospital; ** based on intraoperative findings; 4. tumor necrosis factor α; 5. interleukin1β; 6. inteleukin 6; 7.interlukin 8; *** treatment with IV. lidocaine in constant rate infusion.

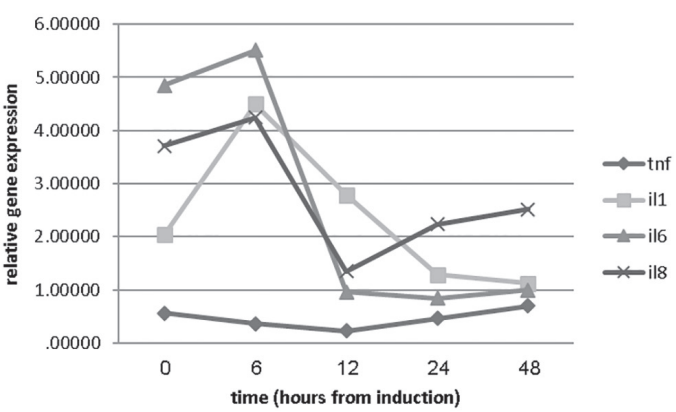

Figure 1: Relative gene expresion of cytokines (TNF-α, IL-1β, IL-6, IL-8) over the time in horses undergoing colic surgery

of the disease confront pro-inflammatory effects and in so doing limit the inflammatory response (6). In case of failure, pro-inflammatory states will predominate and may lead to severe complications (6). In this study, we have followed the dynamics of expression of TNF-α, IL-1β, IL-6 and IL-8 which have been previously shown to have a role during sepsis and surgical interventions (23, 24, 25).

In the present study, levels of TNF-α were decreased from the first sampling before surgery at the induction of anesthesia continuing to decrease over the next 12 hours and then gradually increasing over time and staying below reference range even after 48 hours in all horses. TNF-α was the first cytokine to be detected after exposure to LPS

(26). In experimental studies, TNF-α reach peak expression 60 minutes after exposure to LPS, returning to basal levels at 180 minutes and kept decreasing due to initiation of the production of anti-inflammatory cytokines in the latter stage of disease (1, 14, 26, 28, 29). It acts as a potent activator of neutrophils by mediating their adherence, chemotaxis and degranulation, by vasodilatation and increased vascular permeability and negative inotropic effects during toxic and septic sepsis (27). Expression of TNF-α can peak early during an inflammatory response and can decrease rapidly after 1 to 2 hours, even during continuous infusion of LPS and persistence of fever (14, 28, 29). Decreased expression of TNF-α seen in this study indicates the relatively early stage in the activation of inflammation and correlates with the decreasing phase of TNF-α.

In contrast to TNF-α, the IL-1β levels were elevated from the first sampling before surgery and 6 hour later, decreasing over time and reaching reference values after 48 hours. Interleukin-1 beta is a key mediator of acute inflammatory response to microbial invasion, inflammation, immunological reactions, fever and tissue injury (27). IL-1β production may be stimulated by a variety of agents, including endotoxin, other cytokines, primarily TNF-α, microorganisms, and antigens (27). One of the most important biological activities of IL-1β is its ability to activate T lymphocytes by enhancing the production of IL-2 and expression of IL-2 receptors (27). In addition, IL-1β elicits the release of histamine from mast cells at the site of inflammation (30). Moreover, TNF-α and IL-1β act in synergism in the initiation phase of the inflammatory reaction (31) sharing numerous biological activities. Again, results of this study points on activation of IL-1β by TNF-α in the early proinflamatory stage and endotoxemia.

Similarly, levels of the IL-6 were increased on the first sampling before surgery and 6 hour later. It then started to decrease being slightly below reference interval after 48 hours from the time of the first sampling. IL-6 expression is in part under the control of several endogenous pyrogens well as by IL-1β and TNF-α (32). The members of this cytokine family have pro- as well as anti-inflammatory properties and are major players in acute-phase and immune responses of the organism. Under the influence of IL-6, B lymphocytes differentiate into mature plasma cells and secrete immunoglobulins with T-cell activation, growth, and differentiation (23). In contrast to these pro-inflammatory effects, it shares anti-inflammatory effects such as down-regulation of the inflammatory cascade by inhibition of TNF- α synthesis (27, 32). Well established production of IL-6 in the first measurement in this study can explain the low levels of TNF-α production and its decreased expression.

Levels of IL-8 were increased on first sampling before surgery, peaking after 6 hours and then decreasing over time and staying elevated compared to reference value after 48 hours. Interleukin-8 stimulates polymorphonuclear leukocyte function by attracting these cells to sites of inflammation and inducing expression of cell surface markers (30). It belongs to a chemotactic cytokine family and it is responsible for the chemotactic migration and activation of neutrophils and other cell types (such as monocytes, lymphocytes, basophils, and eosinophils) at sites of inflammation (30). Synthesis of IL-8 is stimulated by TNF-α and IL-1β (32). This cytokine is been used in the prognosis of critically ill patients (34, 35) by itself or in combination with other inflammatory cytokines such TNF-α, IL-1β an IL-6 (36). However in this study no correlation was found between IL-8 and clinical presentation or postoperative recovery from colic surgery.

Lidocaine has been shown to have anti-inflammatory properties in the course of endotoxemia, due to a decrease in production of pro-inflammatory cytokines (37, 38, 39, 40, 41). In order to achieve this effect, lidocaine was given in experimental and clinical studies before or immediately after induction of endotoxemia (37, 38, 39, 40, 41). Recently experimental LPS injection to horses followed by a bolus of lidocaine and constant rate infusion has shown that horses treated with lidocaine had significantly lower clinical scores and serum and peritoneal fluid TNF-α activity (39). In the present study there was no significant difference in the measured cytokines between lidocaine treated and non- lidocaine treated horses. We assume that the timing of lidocaine administration is crucial for its efficacy. In the present study horses were already in advanced stages of the clinical and inflammatory process in contrast to the above mentioned experimental study (39) in which lidocaine was administrated 20 minutes after the induction of the endotoxemia. Another two clinical studies evaluating the efficacy of the treatment of lidocaine in canine gastric dilatation and volvulus have shown results that support this theory (42, 43). In the first retrospective study there was no difference in any clinical, complication and outcome between treatment and non-treatment groups (42), while in the second study there was significant reduction in the complication occurrence (e.g

arrhythmias and acute kidney injury) (43). It was concluded by the authors of these two studies that the rate and timing of the lidocaine administration was probably the reason for the lack of influence in the first study (42).

This study has a few limitations. First, the number of the horses was not large enough to reach any statistical significance between treatment groups. Secondly, the retrospective nature of the study and lack of data made the analysis weaker and less significant. In the present study the presence of endotoxemia was not evaluated by specific biomarkers (e.g LPS). However, in all the horses in the present study changes in the cytokine were noted in most of the cases, emphasizing the inflammatory reaction in equine colic with or without endotoxemia. Finally, screening was done mainly for pro-inflammatory cytokines and inclusion of anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 may contribute to better understanding of the dynamic of the inflammatory process.

In conclusion, in this study we present the dynamics of the expression of inflammatory cytokines over time in a clinical setting of horses with colic. Cytokine production was uniform and confirms that all acute abdominal diseases in horses have similar pathophysiology, regardless of its primary etiology. Decreased levels of TNF-α together with elevated levels of Il-1, IL-6 and IL- 8 upon admission suggest a late intervention in the course of inflammation. Further, larger scale studies are needed to gain a better understanding of the dynamics of cytokine in horses with colic production in order to established prognostic and treatment guidelines.

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