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Research article

Activation of inflammatory cytokines and oxidative stress of horse immune response after cobra venom immunization for therapeutic antivenom production

Sunutcha Suntrarachun[a,*], Kanyanat Promruangreang[a], Suchitra Khunsap[a], Surasak Akesowan[b]

[a] *Research and Development, Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok 10330, Thailand*

[b] *Horse Farm Laboratory and Animal Breeding Center, Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok 10330, Thailand*

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Abstract

Importance of the work: Inflammatory cytokines and oxidative stress provide useful information relating to the immune response of horses after cobra boosting.

Objectives: To evaluate the release of inflammatory cytokines and oxidative stress in horses after receiving a cobra booster.

Materials & Methods: Twenty horses used for producing cobra antivenom were separated into three groups of immune phenotypes according to neutralizing antibody titer in mice (high antibody titers, low antibody titers and non–response). Cytokine mediators, related proteins and oxidative stress were measured up to 1 mo after a cobra booster using SYBR Green real-time reverse transcriptase polymerase chain reaction and biochemical methods, respectively.

Results: Only horses with high and low antibody titers began to elevate substantially on d 7 and peaking on d 14 but at different levels. Oxidative stress, malondialdehyde, glutathione reductase, catalase and superoxide dismutase, with high and low antibody titers, were significantly ($p < 0.05$) increased on d 7 after getting a booster dose and were only slightly changed in horses in the non-response group. Only IL–4 and IL–10 showed significant ($p < 0.05$) differences under the influence of the three groups of immune phenotypes and date–time.

Main finding: With a cobra booster, measurement of mRNA expression of cytokine mediators (especially IL–4 and IL–10) and oxidative stress could be additional immunological parameters to monitor or predict clinical responses to cobra venom and may also reduce the number of animals used in the process of snake antivenom production. The gained knowledge should help to develop strategies to achieve a successful immunization schedule and high–quality snake antivenom.

* Corresponding author.
E-mail address: sunutcha@yahoo.com (S. Suntrarachun)

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Introduction

Snakebite envenomation is an important public health problem worldwide (Leon et al., 2011). Snake venom contains different components of proteins, enzymes and toxins; a snake venom injection can be related to simultaneous processes, including toxic effects of the snake venom and the stimulation of the immune response of host to neutralize the components of snake venom (Da Silva and Tambourgi, 2011). The toxic effects of snake venom depend on the physicochemical properties of the venom, such as the relative abundance of various components, their structure and the molecular weight of the venom components (Leon et al., 2011). The immune response against snake venoms relies on host factors, such as the individual genetic background of the immunized hosts and the capacity of the host immune response to recognize the venoms (Angulo et al., 1997; Leon et al., 2011). Antivenoms are considered to be the only effective treatment for snake envenomation; however, snake venom antiserum produced from some horses in many countries, including Thailand, has been frequently of low potency, leading to antivenom shortages (Potet et al., 2021). The difficulty in producing potent antivenoms is the generation of antibody response to recognize and neutralize the venom components. Many factors influence antibody response against snake venoms in animal hosts. These include intrinsic host factors (age, sex and genetic background) and extrinsic factors (preexisting immunity, infections, and antibiotics) (Leon et al., 2011). Furthermore, environmental factors (geographic location, season and toxins), behavioral factors (exercise and sleep) and nutritional factors (such as body mass index and micronutrients) influence how individuals respond to immunization, while immunization factors (adjuvant and dose) and administration factors (schedule, site and route) are also important (Leon et al., 2011). Understanding of these factors should lead to experimental designs of immunization schedules to improve snake venom immunogenicity and enhance the immune response.

Cytokines are small proteins that play an important role in the immune system by controlling the activation, proliferation and differentiation of T and B–lymphocytes (Da Silva and Tambourgi, 2011). Therefore, the characterization and quantification of cytokine release are essential for understanding the regulation of the immune process. Cytokines act as immunological markers to perceive the pathogenesis of the infectious diseases, inflammatory reactions and organ transplant rejection (Forlenza et al., 2012; Sanchez–Matamoros et al., 2013). Several important cytokines, such as IL–4, IL–10, Interferon-gamma (IFN–g), tumor necrosis factor (TNF–a) and proteins related to T and B–lymphocytes, such as CD19, E2A and Forkhead Box P3 (FOXP3) are involved in the immune response to infection and immunization (Jalali et al., 2015). A variety of methods are used in a wide variety of protein expression profiling applications, including diagnostic and basic immunological research in humans and animals (Domingos et al., 1990). Quantitative real time reverse transcriptase-polymerase chain reaction (RT–PCR) is the method of choice used to quantify the mRNA expression of cytokines which are often expressed at low levels (Sanchez–Matamoros et al., 2013). The release of inflammatory cytokines can induce an oxidative stress response which reflects the imbalance between the levels of reactive oxygen species (ROS) and antioxidants (Santhosh et al., 2013). ROS are derivatives of oxygen and involved in the inflammatory responses (Salman and Hammad, 2017). ROS play essential roles in cell physiology and participate in the pathological conditions (Salman and Hammad, 2017). The free radicals apart from being involved in cell damage can play a significant role in venom toxicity (Santhosh et al., 2013; Al–Quraishy et al., 2014). Chemical and environmental toxins cause the production of ROS that play an important role in the progression of inflammation (Santhosh et al., 2013; Salman and Hammad, 2017). Many studies have indicated that snake venom also causes overproduction of ROS with oxidative damage and inflammatory responses in animals (Santhosh et al., 2013; Al–Quraishy et al., 2014; Tohamy et al., 2014). Detoxification of ROS can be achieved by an efficient antioxidative system comprising nonenzymatic antioxidants such as ascorbic acid, glutathione phenolics and lipid peroxidation (malondialdehyde, MDA) as well as enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GSH) (Ohkawa et al., 1979; Pajovic et al., 1997; Tipple and Rogers, 2012; Salman and Hammad, 2017).

A large percentage of the horses used for cobra antivenom production produce low antibody titer and a non–response against cobra venom (Akesowan et al., 2015). Therefore, the current study aimed to determine the effect of snake venom on inflammatory cytokine gene expression in peripheral blood mononuclear cells (PBMC) and plasma oxidative stress markers after receiving a booster dose in horses used for cobra antivenom production. All horses were classified according to the neutralizing antibody titer

in mice, with the three groups being high antibody titer, low antibody titer and non–response. The pattern of gene expression of inflammatory cytokines and venom-induced oxidative stress should provide useful information for improving strategies to achieve the most successful immunization schedule. Furthermore, this would help in the selection of suitable horses to obtain high antibody titer for antivenom production. Then, the high-quality snake antivenoms produced could be made available to people in Southeast Asia and worldwide.

Materials and Methods

Horse

The 20 horses used in the study were from routine immunization against cobra venom for cobra antivenom production. They were aged 3–4 y, weighing 450–550 kg and were gelded males. The antibody titer (potency) was checked using a neutralization test in mice with the cutoff value of 0.26 mg/mL to select horses for the plasmapheresis process. All horses had been rested for about 2 mo after the plasmapheresis process before receiving a booster dose. The animal protocol for collecting ethylenediaminetetraacetic acid (EDTA) blood samples was reviewed and approved by the Queen Saovabha Memorial Institute Animal Care and Use Committee (QSMI–ACUC–06–2018).

Immunization protocol

Prior to receiving a booster dose, the 20 horses were classified into three groups according to the neutralizing antibody titer in mice. The first group (high antibody titer) comprised six horses passing the cutoff (0.26 mg/mL) and neutralization test in mice (survival rate > 60%). The 12 horses in the second group (low antibody titer) did not pass the cutoff and had survival rates in the range 20–60%, while the third last group (non-response) contained two horses with non-response to the immunization and so did not pass the cutoff antibody titer and had 0% survival rate. All horses had received a booster dose using 20 mg/mL cobra venom that was well mixed 1:1 with Alum adjuvant (Imject Alum; Pierce; Franklin, MA, USA) to form an emulsion. The concentration of cobra booster dose and Alum adjuvant came from the last dose of the routine immunization schedule for cobra antivenom production.

At least 20 mL of EDTA blood samples were taken per horse prior to the booster dose (d 0) and on d 1, 3, 7, 14, 21 and 28 following the booster dose. Individual measurements of horses were used and not the pooled samples. All samples were measured in triplicate. Repeat measurement on different days was carried out for the independent experiments.

Enzyme-linked immunosorbent assay for antibody response

The plasma antibody response of the horses in the three groups of booster were evaluated using enzyme–linked immunosorbent assay (ELISA). Plates of 96 wells (Nunc Inc.; USA) were coated with 2.5 mg/mL cobra venom and left standing overnight at 4 °C. Then, the plates were washed five times with washing buffer. The remaining binding sites were blocked with 2% bovine serum albumin in phosphate buffer saline (PBS) with Tween 20 for 2 h at 37 °C. Afterward, the plates were washed again five times with washing buffer. Next, the diluted horse plasma (1:1,000) was added to the plates (50 µL/well), mixed thoroughly before transfering to the next row (1:4) and incubated for 1 h at room temperature. The plates were washed again five times and 1:10,000 anti-horse IgG–peroxidase conjugate was added and incubated at room temperature for 1 h. Next, the plates were washed five times and the substrate was added. Finally, the reaction was stopped using 0.5 M sulfuric acid for 10 min and the absorbance was obtained at 490 nm using an ELISA plate reader. The optimal cut off for ELISA was set at 0.0676.

Lipid peroxidation assay (malondialdehyde)

Malondialdehyde (MDA) is an end product of lipid peroxidation (measured in nanomoles per millimeter) that is considered a late biomarker of oxidative stress and cellular damage (Ohkawa et al., 1979). Induction of oxidative damage was ascertained by measuring the extent of lipid peroxidation in the plasma sample by estimating thiobarbituric acid (TBA) reactive substances according to the method of Ohkawa et al. (1979). Horse plasma (100 µL) was added into 2 mL of 0.37% TBA in 0.25 N HCl and 15% Trichloroacetic acid (TCA) in 0.25 N HCl (1:1) and boiled for 10 min at 90 °C. The solution was cooled at room temperature, centrifuged at 12,000 revolutions per minute (rpm) for 10 min and the supernatant kept. Then, the solution was measured for absorbance at 532 nm.

Determination of reduced glutathione

The reduced glutathione (GSH) levels were determined using a fluorimetric method described by Tipple and Rogers (2012) with minor modification (Tipple and Rogers, 2012). To 100 µL of horse plasma was added an equal volume of 20% TCA containing 1mM EDTA (for protein precipitation). The mixture was incubated at room temperature for 5 min and centrifuged at 2,000 rpm for 10 min. Then, 200 µL of supernatant was transferred to a new tube containing 1.8 mL of 0.1 mM 2,4–dinitrothiocyanatebenzene (DNTB), 0.1 PBS and 1% sodium citrate. The absorbance of the solution was measured at 412 nm.

Catalase activity

The Catalase CAT activity was determined by measuring the rate of hydrolysis of H_2O_2 at 240 nm (Johansson and Borg, 1988). Horse plasma (100 µL) was added in 1.9 mL of PBS pH 7.2. Then, 1 mL of 30 mM H_2O_2 was added to start the reaction and the solution was mixed thoroughly. The absorbance of the solution was measured at 240 nm every 15 s for 3 min at room temperature. The catalase activity was calculated from a catalase standard (measured in units per milliliter).

Activity of superoxide dismutase

Superoxide dismutase (SOD) activity is one of the most important antioxidative enzymes; it catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen and the rate of the reduction with a superoxide anion is related to the xanthine oxidase activity and is inhibited by SOD (Pajovic et al., 1997). Therefore, the inhibition activity of SOD can be determined using a colorimetric method (Pajovic et al., 1997). Horse plasma (50 µL) was added in 2.9 mL of solution A (5 µmol of xanthine in 10 mL 0.001 N NaOH) and mixed with 50 µL of solution B (xanthine oxidase in 0.1 mM EDTA) to start the reaction. Then, the solution was measured for absorbance at 550 nm every 1 min for 4 min at room temperature and reported as units per milliliter.

Cytokine and protein coding genes primers

Oligonucleotide primers of IL–1b, IL–10, IFN–g, TNF–a, IL–4, CD19, E2A, FOXP3 and b–actin were designed based on the sequences of horse cytokines deposited in the GenBank database (Table 1.), with b–actin used as a housekeeping gene for normalization. Primer sets were developed and optimized using Primer 3 based on the default parameters. Nine PCR primer pairs were selected using the same criteria, including an annealing temperature of 52 °C, primer length 18–24 bp and PCR product size 100–400 bp.

Table 1 Oligonucleotide primers of IL–1b, IL–10, IFN–g, TNF–a, IL–4, CD19, E2A, FOXP3 and b–actin based on GenBank database

Primer	Nucleotides (5’ ® 3’)	Product Size (bp)	GenBank database
IL1b–F	5’TGTACCTGTCTTGTGGGATGAAA 3’	184	NM001082526
IL1b–R	5’ TTCTGCTTGAGAGGTGCTGA 3’		
IL10–F	5’ GTCATCGATTTCTGCCCTGT 3’	180	NM001082490
IL10–R	5’GCTTCGTTCCCTAGGATGC 3’		
IFNg–F	5’ TGGACACCATCAAGGAGGAC 3’	107	EU000433
IFNg–R	5’GGACCTTCAGATCATTTACCG 3’		
TNFa–F	5’ TTACCGAATGCCTTCCAGTC 3’	271	M64087
TNFa–R	5’ GGGCTACAGGCTTGTCACTT 3’		
IL4–F	5’ CAAAACGCTGAACAACCTCA 3’	247	AF305617
IL4–R	5’TGCTCTTCTTGGCTTCATTC 3’		
CD19–F	5’ CCAGTCACCAGGACAACAGA 3’	396	JN979558
CD19–R	5’ GGCTGAAGTTTCGCTCATGT 3’		
Predicted E2A–F	5’ GCTGCACCTCAACAGTGAGA 3’	152	XM001915704
Predicted E2A–R	5’ CCGACACCTTCTCCTCTTCC 3’		
FOXP3–F	5’ CTTTCACAACCAACCACACA 3’	194	EU554426
FOXP3–R	5’ TGTGTTCAGGCATGATTGTC 3’		
b–actin–F	5’ACCAACTGGGACGACATGGACAA3’	380	NM001081838
b–actin–R	5’ GTGGTGGTGAAGCTGTAGCC 3’		

Cytokine mRNA expression using SYBR Green real-time reverse transcriptase-polymerase chain reaction

Peripheral blood mononuclear cells (PBMC) of the horse samples were separated using Ficoll-Paque™ Plus (GE Healthcare; Uppsala, Sweden). Total RNA was extracted from horse PBMC using 500 µL of TRIzol Reagent (Molecular Research Center Inc.; Cincinnati, OH, USA), according to the manufacturer's instructions. The concentration and purity of the extracted total RNA were determined by measuring the absorbance ratio at wavelengths 260 nm over 280 nm using a spectrophotometer. Quantitative SYBR Green real-time RT–PCR was carried out with Light Cycler (BioRad CFX 96 Touch™ Real Time PCR; Berkeley, CA, USA). The expression levels of the cytokines and protein coding genes in the horses were estimated using an iTaq Universal SYBR Green One–Step Kit (BioRad; Berkeley, CA, USA), according to the manufacturer's instructions. The reaction was performed under the following conditions: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 52 °C for 10 s and finally 72 °C for 30 s. The melting curve analysis was performed following the amplification to verify the specificity of the PCR product by looking at melting temperatures. The melt curve protocol was followed by 95 °C for 10 s and then by 0.5 °C increments for 5 s each from 65 °C to 95 °C, with b-actin used as a housekeeping gene to amplify under the same conditions as the protocol above. The evaluation of the gene expression level was based on the comparative threshold cycles (Ct) method also referred to as the $2^{-\alpha a\,Ct}$ method (Ramos-Payan et al., 2003; Forlenza et al., 2012; Sanchez-Matamoros et al., 2013, Akesowan et al., 2015).

Statistical analysis

All experiments were performed in triplicate and repeated measurements were from different independent experiments. Two-way analysis of variance (ANOVA) was performed with two factors immune phenotypes, high antibody titer, low antibody titer and non-response) and date-time (d 0, 1, 3, 7, 14, 21 and 28). Tukey multiple comparisons test was employed for mean comparisons. All tests were considered statistically significant at $p < 0.05$.

Results

Enzyme-linked immunosorbent assay for antibody response

The absorbance value at 490 nm of the normal horse plasma in developed ELISA for cobra venom was 0.0313 ± 0.0363 and the cutoff value was 0.0676. Sample values measured at 490 nm that were less than or equal to the cutoff point were considered negative results. The only significant differences were between dates and different groups. The plasma antibody levels in the three groups of booster horses were evaluated. The results of ANOVA revealed significant effects on groups of immune phenotype as well as date-time ($F = 6.41$ and 10.78; $p < 0.05$), while the interaction was non-significant ($p > 0.05$). The ELISA titer was highest for the group of high antibody titer followed by low and non-response, respectively (Table 2). For date–time, the ELISA titer remained unchanged from d 0 to d 7. The significant increase was seen at d 7 and the highest peak was seen at d 14 (Table 3). The least squre mean of ELISA titer for each treatment combination are shown in Fig 1.

Table 2 Least square mean ± SE of ELISA titers measured at d 0 to d 28 after immunization averaged across three immune phenotypes of horses

Day	Mean ± SE
0	0.47 ± 0.08^{c}
1	0.46 ± 0.08^{c}
3	0.60 ± 0.08^{c}
7	0.82 ± 0.08^{ab}
14	1.05 ± 0.08^{a}
21	0.64 ± 0.08^{bc}
28	0.55 ± 0.08^{c}

Mean ± SE superscript with different lowercase letters denote significant ($p < 0.05$) differences between groups

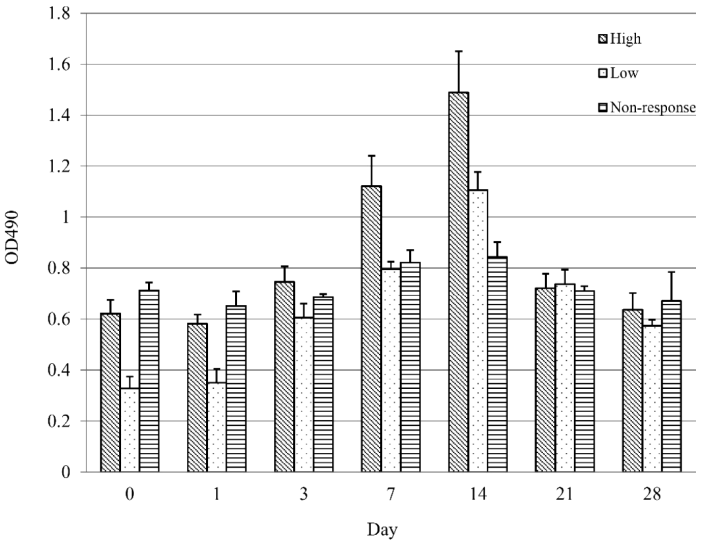

Fig. 1 Antibody titers from three groups of horses (high, low and non-response) before (d 0) and after being immunized with cobra venom measured at different consecutive date-time, where histograms represent mean ± SD of each group.

Oxidative stress: Malondialdehyde, glutathione reductase, catalase and superoxide dismutase

There were significant differences in the oxidative stress for booster horses between d 3 and d 28. Plasma MDA levels in the groups of high and low antibody titers increased sharply after immunization, peaking on d 7 and were higher than those in the non-response group. The plasma GSH levels among the three groups of horses started to increase significantly on d 3 after booster immunization and reached a peak on d 7 before dropping slowly. The pattern of plasma CAT levels increased significantly on d 3 and reached its peak on d 7 before suddenly declining to a lower and more stable level from d 14–28. In addition, the plasma SOD levels were strongly and significantly elevated on d 3 and peaked on d 7; however, after that they suddenly declined until returning to a normal level. The pattern of plasma SOD levels and CAT levels among the three groups were similar but at different levels. The two–way ANOVA examined the effect of immune phenotype and date–time on the levels of oxidative stress for MDA, GSH, CAT and SOD. There were significant differences in the levels of oxidative stress between the effects of immune phenotypes, date–times, and interaction (Fig 2.).

Cytokine mRNA expression using SYBR Green real-time reverse transcriptase–polymerase chain reaction

SYBR Green real-time RT–PCR was used to analyze mRNA gene expression of cytokines and related proteins in the EDTA of horse blood after the horse had received a booster dose with cobra venom for d 1, 3, 7, 14, 21 and 28. The concentration of optimized primer sets was 10 mM/µL. All primer sets had the same annealing temperature (52 °C) and were run in parallel in the same real–time thermo cycler. The melt curve protocol was 95 °C for 10 s and then 5 s at each 0.5 °C increment from 65 °C to 95 °C. The concentration of extracted total RNA was 1 µg. Data analysis was obtained in 2 h hence, it was less time–consuming.

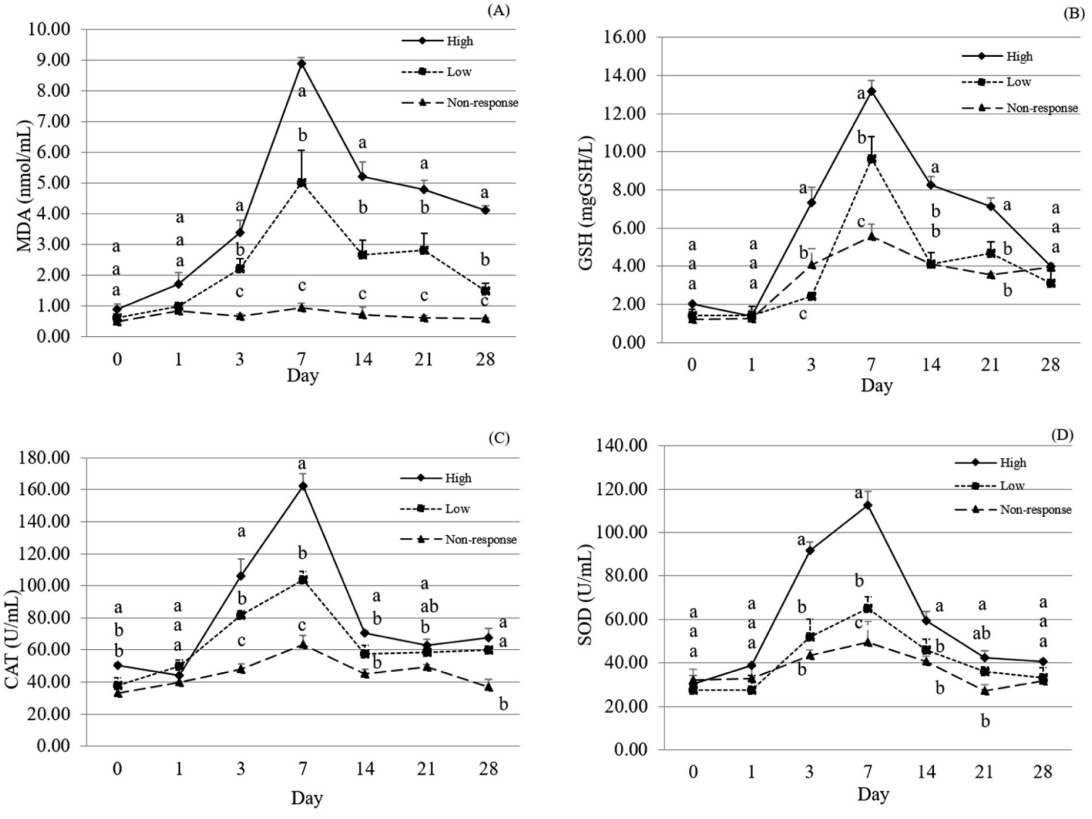

Fig. 2 Oxidative stress evaluated in three groups of horses (high, low and non-response) immunized with cobra venom before and after receiving a cobra booster: (A) lipid peroxidation (MDA); (B) reduced glutathione (GSH); (C) catalase activity (CAT); (D) superoxide dismutase (SOD), where results are mean ± SD of three groups of horses (high, low and non-response). Lowercase letters denote significant ($p < 0.05$) difference between immune phenotypes of each date–time.

There are statistically significant differences, $p < 0.05$, between three immune phenotypes and date-time of IL–4 and IL–10 (Figs. 3A and 3B). Gene expression levels in the groups of high and low antibody titers for IL–4 and IL–10 increased ($p < 0.05$) between 2–fold and 3–fold as early as 24 h after receiving a booster. Peak expression levels were observed on d 3 and d 7 for the high and low antibody titers, respectively, and returned to a normal level in the next few weeks. The, non–response group had a small change in the gene expression of IL–4 and IL–10.

The gene expression level of IL–1b in all groups slightly increased by about 1–2–fold for d 14 after a booster dose of cobra venom. The protein coding genes CD19 and E2A were not observed in the mRNA expression until d 28, being 8–fold and 9–fold, respectively, of the horses with high antibody titers. However, horses in the low antibody titer and non–response groups had some slight changes in the PBMC of these markers. The gene expressions of TNF–a and IFN–g in horse PBMC from all groups were relatively down–regulated. Similarly, the expression level of FOXP3 in all groups slightly decreased after receiving a cobra snake booster and returned to a normal level on d 28. However, there were no significant differences, $p > 0.05$, between the three immune phenotypes and date-times of IL–1b, IFN–g, TNF–a, CD19, E2A and FOXP3 (Figs. 3C–3H).

Discussion

The current results demonstrated mRNA gene expression of inflammatory cytokines and other related proteins and oxidative stress after a booster dose in horses routinely used for cobra antivenom production. A significant increase of inflammatory cytokine gene expression and oxidative stress occurred at d 3 before rising of antibody titer at d 7 after a booster dose in horses. The limitations of this study were the small sample size, which limited the power of statistical testing, as only horses with cobra venom immunization for antivenom production were used in this study. Another limitation of the research was the short time period available for blood collection.

Real-time RT–PCR based on SYBR has been recognized as a quick, accurate and relatively inexpensive method to quantify the mRNA expression of cytokine- and protein- encoding genes in veterinary species following immunization (Forlenza et al., 2012). The SYBR Green real-time RT–PCR method described herein should allow characterizing the expression of the cytokine– and protein–encoding genes involved in the immune response in the PBMC of horses following a booster dose. This useful information should help to develop strategies to achieve a successful immunization schedule and high–quality snake antivenoms. The cytokines analyzed were selected for their roles in inflammatory response (IL–1b and TNF–a) and in the activation of cellular immunity (IFN–g) and humoral immunity (IL–4 and IL–10). The immune-related genes CD19, E2A and FOXP3 were also measured in association with B and T lymphocytes (Santhosh et al., 2013; Tohamy et al., 2014). This information is useful in identifying the many factors affecting low level antibody production and non–response. In addition to optimization strategies to achieve the most successful immunization schedule the method can help to produce a potent cobra antivenom.

The booster dose described herein used the same concentration of cobra venom and alum adjuvant as from the last dose of the routine immunization schedule. It is well known that alum adjuvant, which is highly immunomodulatory and forms short-term depots, induces strong Th2 type responses. It was likely that the IL–4 and IL–10 levels significantly increased in the horse PBMC soon after the cobra venom booster in the alum adjuvant, while IL–1b was slowly detected. The mRNA expression levels of IL–4 and IL–10 peaked on d 3 and d 7 in horse PBMC with high and low antibody titers, respectively, after booster with cobra venom. IL–4 and IL–10 are considered important Th2 cytokines that promote B–cell growth and the differentiation of a variety of immune cells against foreign antigens (Leon et al., 2011; Saini et al., 2019). The protein–encoding gene E2A in B cell development generates specific antibodies and B cell–specific genes, such as CD19, induced very similar expression levels of up–regulation in horse PBMC from animals with high antibody titers rather than horses from the low antibody titers and non–response groups (Da Silva and Tambourgi, 2011; Leon et al., 2011). However, a booster dose of cobra venom immunization could not significantly alter the TNF–a and IFN–g levels in the three groups from d 1 through to d 28. It seems likely that the mRNA from these cytokines is best extracted within 24 hr of stimulation (Jalali et al., 2015). It has been shown recently that the mRNA expression of FOXP3 was down-regulated in all groups after venom booster and returned to normal on d 28. It seems likely that down–regulation of the FOXP3 gene is commonly found in inflammatory conditions and is an essential mediator of central tolerance (Angulo et al., 1997; Da Silva and Tambourgi, 2011; Leon et al., 2011). The current results clearly showed that cobra venom could significantly enhance oxidative stress markers, such as antioxidant enzyme

activity, GSH, CAT and SOD. The induction of enzymatic antioxidant defenses after exposure to cobra venom could be considered as an adaptive response to overcome cell damage. To further demonstrate the implication of oxidative stress in a venom–induced toxicity, MDA was monitored as an excellent indicator of free radical production. This fact emphasizes that the oxidative damage is induced by the cobra venom in horse plasma. These results indicated that increased oxidative stress in the plasma after immunization may have functional consequences in terms of a chronic inflammatory response, increased mitochondrial superoxide production and oxidative protein and DNA damage (Domingos et al., 1990; Larson and Wagner, 2021; Liburt et al., 2010; Santhosh et al., 2013; Al-Quraishy et al., 2014).

However, only 20 horses were used in the current study and all of them were from routine immunization against cobra venom for cobra antivenom production. There was a high standard error in the non-response group containing only 2 horses which may not have accurately represented that population of horses used for cobra antivenom production. A low standard error from the low antibody titer group, 12 horses, had a sample mean that was closely distributed around the population mean of the horses. The standard error could be further reduced by increasing the number of horses. Using a large number of horses would be the best way to minimize sampling bias. Furthermore, the results shown in Table 2, Table 3, and Fig 3 could be of concern for factorial linear model analysis, which was influenced by the two variable factors (immune phenotype or date-time) or interaction effects (the combined effects of factors) with two crossed factors.

Immunization of animals to produce snake antivenoms requires the consideration of many factors influencing the immune response towards snake venoms. The relationship among cobra venom injection and cytokine release and accumulated ROS direct contribute to tissue damage and the development of inflammatory response (Domingos et al., 1990). High or low antibody responses in animal hosts show a wide range of diversity which is influenced by inherited or spontaneous genetic variations and environmental effects (Larson and Wagner, 2021). The difference of antibody titers in the individual response of horses to different venoms has been reported. These findings seem likely that immune response to snake toxins is influenced by the genetic individual differences of the animal being immunized (Gutierrez et al., 1988; Angula et al., 1997). Furthermore, the environmental factors might affect the immune system function, including stress, nutrition, climate change and age-related change known as immunosenescence.

Because a large percentage of the horses used for cobra antivenom production have low antibody titer and non–response, their plasma has inadequate neutralizing activity, leading to a shortage of cobra antivenom. The antibody titer (potency) of immunized horses using the neutralization antibody titer in mice was evaluated twice from individual and pooled plasma. The numbers of mice used as models to obtain the antibody titer have been increased. Therefore, cytokine mRNA expression of IL–4, IL–10 and oxidative stress markers could be the additional immunological parameters to reduce the use of animals used in the process of snake antivenom production and also in monitoring and predicting clinical responses to snake venom. Direct monitoring of an individual antibody response against snake venom is expensive in preparing the antibody because it is a sophisticated technique and expensive culture cell media are required to obtain a specific antibody. (Gutierrez et al., 1988; Angulo et al., 1997). The knowledge obtained from the current study should provide useful information for improving strategies to achieve the most successful immunization schedule for high–quality snake antivenom.

Table 3 Least square mean ± SE of ELISA titers measured from three immune phenotypes of horses averaged across date–time

Immune Phenotypes	Mean±SE
High antibody titer	0.84±0.05[a]
Low antibody titer	0.64±0.05[b]
Non-response	0.48±0.05[c]

Mean ± SE superscript with different lowercase letters denote significant ($p < 0.05$) differences between date–times

Conflict of Interest

The authors declare that there no conflicts of interest.

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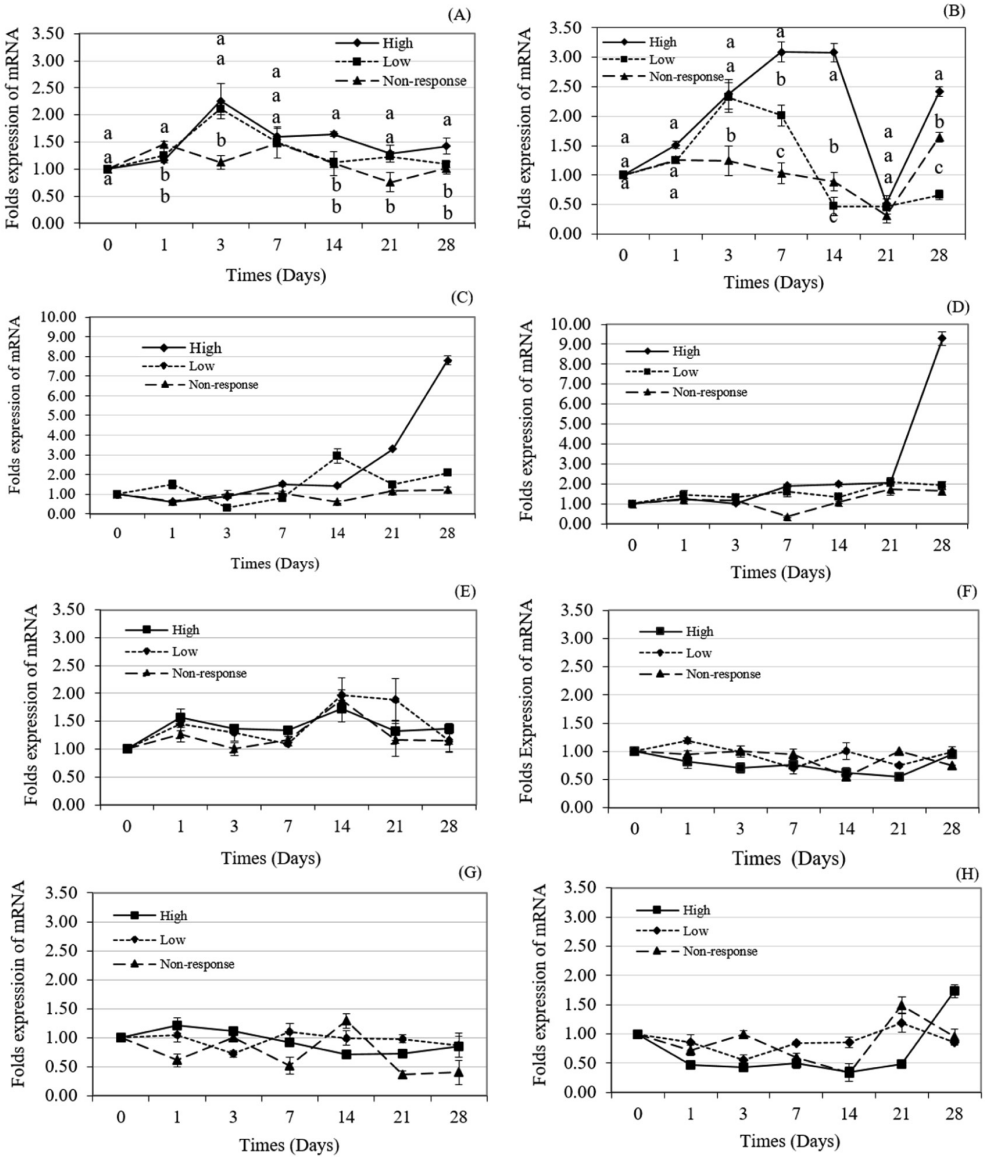

Fig. 3 Measures of relative gene expression in horse blood samples stimulated with a cobra booster at different times of: (A) IL–4; (B) IL–10; (C) CD19; (D) E2A; (E) IL–1β; (F) IFN–g; (G) TNF–α; (H) FOXP3, where results are n-fold difference relative to cytokine mRNA expression of sample untreated and 0 hr has cytokine expression, values are mean ± SD of three immune phenotypes (high, low and non-response). Significant ($p < 0.05$) differences found in main effects (groups of immune phenotype and date–time) and interaction of IL–4 and IL–10.

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