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RESEARCH ARTICLE
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Variations in free radical scavenging activities and antioxidant responses in salivary glands of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* (Acari: Ixodidae) ticks

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Abstract

Aim: *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks are of major economic importance in the livestock sector as the vector of tropical theileriosis causing huge production loss, mostly in tropical countries. The release of different reactive oxygen and nitrogen species by exogenous and endogenous means can potentially induce oxidative damage to the ticks during their prolonged feeding on their vertebrate hosts. Hence, ticks need an effective free radical scavenging and antioxidant defense system for their successful feeding of a blood meal. Therefore, the present study was undertaken to evaluate the interspecies variations in antioxidant response, free radical scavenging, and anti-inflammatory activities in salivary gland extracts (SGE) of the two species as they differ considerably in relation to feeding behavior and host specificity.

Materials and Methods: Tick salivary glands were dissected out under ice from semi-fed female ticks of both the species and homogenized at low temperature to prepare SGE. SGE was stored at −40°C for analysis of free radical scavenging activities and antioxidant status.

Results: Significant depletion in reduced glutathione concentrations, malondialdehyde level and elevation in free radical scavenging activity, superoxide dismutase, anti-inflammatory activity were found in SGE of engorging female *H. dromedarii* ticks as compared to *H. a. anatolicum.*

Conclusion: Higher antioxidant status and free radical scavenging activities in *H. dromedarii* might have enabled these ticks to suck more blood from the host in spite of continuous host's immune responses. These findings about tick biology will help in improving tick control strategies.

Keywords: *anti-inflammatory, antioxidants, free radicals, Hyalomma anatolicum anatolicum, Hyalomma dromedarii.*

Introduction

Ticks are obligatory ectoparasites having wide host range and worldwide distribution. Ticks are capable of transmitting various pathogens to human, as well as livestock animals during their prolonged blood feeding from respective vertebrate hosts. *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks are capable of transmitting *Theileria annulata* into bovines and camels causing tropical theileriosis. It affects milk production, weight loss along with increased mortality and loss in leather production. Tick bite enhances leukocyte migration and phagocytosis at the attachment site(s) generating large amounts of superoxide as part of the mechanism by which foreign organisms are killed [1] by the host. Other mechanisms of the host which are responsible for oxidative stress to the ticks are release of inflammatory mediators, formation of peroxides by activation of lipoxygenases, cyclooxygenase enzymes, release of metal ions from storage sites (iron, copper), generation of free radicals in terms of reactive oxygen species and reactive nitrogen species (ROS and RNS), release of heme proteins [2] and interference with tick antioxidant defense systems. As attachment and feeding takes several days to complete for ixodid ticks, with majority of the blood meal is not taken up until the last day of attachment [3] it provokes strong hemostatic, inflammatory, and immune responses [4] by the host which is the major source of exogenous oxidative stress to ticks.

Besides this, reproduction and metabolic activity like cellular respiration [5] produce endogenous mediators of oxidative damage. Thus, detoxification of ingested heme as well as the scavenging and detoxification of the exogenously and endogenously produced oxidative stressors are essential for survival of ticks. Salivary cocktail of various immunomodulators, antioxidants, anticoagulants, and other biologically active proteins are the major components of tick antioxidant defense system to combat these oxidative assaults [4,6].

Therefore, the present study was planned to evaluate the scavenging capacity for different free radicals such as nitric oxide and hydroxyl radical, level of lipid peroxidation (LPO), anti-inflammatory activity, enzymatic actions of superoxide dismutase (SOD) and nonenzymatic antioxidant reduced glutathione (GSH) in *H. a. anatolicum* and *H. dromedarii* salivary glands to establish a physiological link between the level of enzymatic and nonenzymatic components of tick antioxidant defense system with their role against oxidative stresses imposed by host.

Materials and Methods

Ethical approval

This is a part of MVSc. Thesis work of the first author and the research experiments were conducted after approval of Institutional Animal Ethical Committee (IAEC).

Collection of ticks

Partially fed adult female *H. a. anatolicum* and *H. dromedarii* ticks were collected from buffaloes and camels from the villages around Hisar district of Haryana, India. Six groups were made in each case from randomly selected ticks; each group consisted of six partially fed adult ticks.

Dissection of ticks and collection of salivary glands

The female ticks were glued to the bottom of a Petri dish with their dorsal surface upward and placed on ice for 20 min. Using fine scalpel blade and fine tip forceps, ticks were incised along the dorsal-lateral margin, and the dorsal integuments were removed under a stereoscopic dissection microscope (Magnus MSZ-TR Trinocular Version). Salivary glands were removed as described by Wu *et al*. [7] and were transferred and preserved into preservation buffer (pH 6.0) containing 0.1 M phosphate buffer saline (PBS), 5% glycerol, protease inhibitor (Sigma, P2714) for estimation of antioxidants and anti-inflammatory activities. For the estimation of minerals, the organs were preserved in normal saline solution.

Extract preparation

The salivary glands from ticks were homogenized under ice using a tissue homogenizer (IKA T10 basic Ultra-Turrox) in 0.1 M PBS, pH 6.0, containing protease inhibitor cocktail. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. Then the supernatant was filtered through 0.22 µ syringe filter (MILLEX-GV) and the salivary gland extract (SGE) stored at −40°C for further analysis.

Free radical scavenging and antioxidant activity assays

Nitric oxide radical scavenging activity

Nitric oxide scavenging activity was measured in SGE as described by Sreejayan and Rao [8]. For this, different concentrations of fresh extracts in standard phosphate buffer were incubated with 5.0 mM sodium nitroprusside at 25°C for 3 h. After incubation, 0.5 ml of the incubated solution was removed and mixed with an equal volume of Griess reagent. The absorbance of the chromophore was taken at 546 nm against blank without sodium nitroprusside, using ultraviolet-visible (UV-VIS) spectrophotometer (thermo scientific Multiskan spectrum-UV/VIS microplate and the cuvette spectrophotometer). The samples were analyzed in triplicate.

Scavenging of hydroxyl radical

Hydroxyl radical scavenging activity was measured in SGE as described by Halliwel *et al*. [9]. Reaction mixture contained 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM H_2O_2, 0.1 mM ascorbate and different concentrations of extract in a final volume of 1.0 ml. 0.5 ml of thiobarbituric acid (TBA) and 0.5 ml of ice-cold trichloroacetic acid (TCA) was added to the reaction mixture followed by incubation at 37°C for 1 h. Absorbance was measured at 532 nm. Reaction mixture containing an equal volume of buffer instead of sample was used as control.

SOD assay

The activity of SOD in SGE was measured as described by Madesh and Balsubramanium [10]. For the estimation of SOD activity, 0.65 ml of PBS and 30 µl of 1.25 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each of the tubes marked as the sample, control, and blank. 10 µl of tissue homogenate was added to the sample tube. After that 75 µl of 100 µM pyrogallol was added to all the tubes and incubated for 5 min at room temperature. 0.75 ml of dimethyl sulfoxide was added in all the tubes to terminate the reaction, and 10 µl tissue homogenate was added to the control tube. The absorbance was measured at 570 nm. The activity of the SOD was calculated as follows:

SOD (units/mg of protein) = (mg of protein in 0.01 ml homogenate/y) × 50 × DF

$y = ([Absorbance_{control}\text{-}Absorbance_{sample}]/Absorbance_{control}) \times 100$

(y = % inhibition of MTT reduction by SOD protein)

(DF = Dilution factor)

Reduced GSH assay

The concentration of GSH was estimated as described by Beutler [11]. 500 µl of supernatant homogenate in phosphate buffer (pH-7.4) containing 0.02 M EDTA was mixed with 400 µl of distilled water and 100 µl of 50% TCA solution. The contents were incubated at room temperature for 20 min and centrifuged at 3000 rpm for 15 min. 800 µl of supernatant was taken and 1 ml of 1 M Tris-HCl (pH-8.0) buffer was added to it. Then 80 µl of DTNB reagent (0.14 M sodium chloride, 0.009 M disodium hydrogen phosphate and 40 mg/100 ml 5,5'-dithiobis [2-nitrobenzoic acid]) was added and kept in reducing condition at room temperature for 15 min. The absorbance was measured at 412 nm. A standard curve of GSH

was prepared using concentrations ranging from 0.02 to 0.1 mM of GSH in 0.02 M EDTA, and the values of the samples were extrapolated from that curve.

LPO assay

LPO levels were determined using the method of Ohkawa *et al*. [12] in which the malondialdehyde (MDA) release served as the index of LPO. MDA contents were determined by measuring the TBA reactive species after incubation at 95°C with TBA (1% w/v) for 60 min. The absorbance of the resultant pink product was measured at 532 nm. Different concentrations of 1,1,3,3-tetramethoxypropane were used as a standard.

Estimation of in-vitro anti-inflammatory activity

The anti-inflammatory activity was determined according to the method described by Shinde *et al*. [13] with some modifications. Blood was collected from healthy buffaloes. The collected blood was mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% sodium chloride in water) and centrifuged at 3000 rpm for 15 min. Packed cells were washed with isosaline (0.85%, pH 7.2) and a 10% (v/v) suspension was made. The assay mixture contained the SGE, 1 ml of phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of red blood cell suspension. Instead of hyposaline, 2 ml of distilled water was used in control tubes. All the assay mixture were incubated at 37°C for 30 min and centrifuged. The hemoglobin content in the supernatant was estimated at 560 nm. The percentage of hemolysis was calculated using the formula:

% Hemolysis = O.D. of treated sample *100/O.D. of control.

Protein assay

The protein content in the extracts was determined using the method given by Lowry *et al*. [14] using bovine serum albumin as a protein standard.

Statistical analysis

All the data were subjected to statistical analysis for comparing means and correlation among different parameters as described by Snedecor and Cochran [15].

Results

Significant species variations in free radical scavenging activity, as well as antioxidant status, were observed in *H. a. anatolicum* and *H. dromedarii* ticks.

Free radical scavenging and antioxidant activities

Nitric oxide radical scavenging activity

The nitric oxide scavenging activity (Figure-1) increased with the increase in protein concentration in SGE of both the tick species but it did not follow the linear correlation. The activity was significantly higher in SGE of *H. dromedarii* (38.84±1.52-49.81±2.91%) than *H. a. anatolicum* ticks (24.46±1.37-32.17±1.73%) at protein concentrations 3-24 µg/ml.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity (Figure-2) also increased with the increase in protein concentration in SGE of both the tick species and followed a similar pattern. The SGE of *H. dromedarii* was found to have significantly higher activity that ranged from 16.64±0.26 to 27.0±1.05% than *H. a. anatolicum* ticks where the activity ranged from 15.39±0.27 to 21.11±0.38% at the protein concentrations of 3-24 µg/ml.

SOD activity

The species' variations in SOD activities are presented in Figure-3. Significantly higher SOD activity was observed in SGE of *H. dromedarii* ticks (5.75±0.35 units/mg of protein) than *H. a. anatolicum* ticks (4.74±0.66 units/mg of protein).

Reduced GSH depletion

Difference in GSH concentrations between the two tick species is presented in Figure-4 in relation to different protein concentrations (7.5-60 µg/ml)

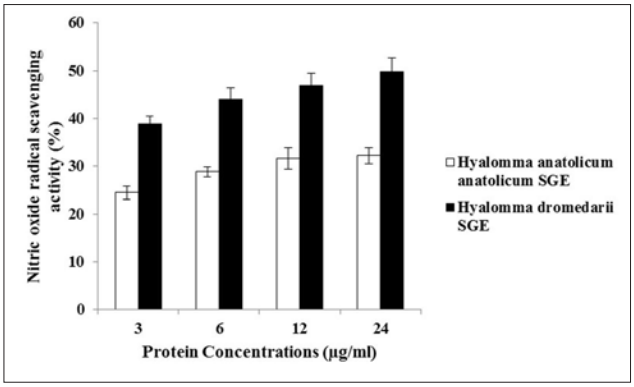

Figure-1: Nitric oxide radical scavenging activity (%) in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 groups, each group containing six ticks.

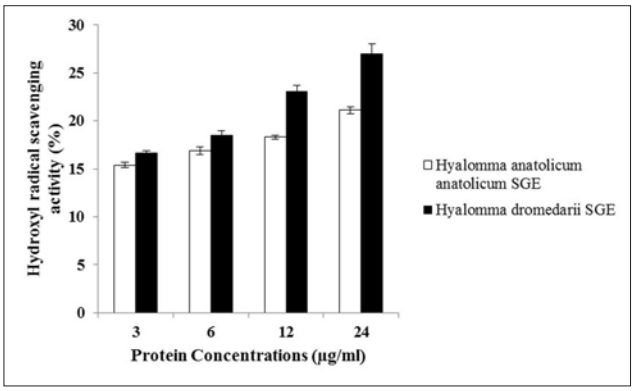

Figure-2: Hydroxyl radical scavenging activity (%) in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 Groups, each group containing six ticks.

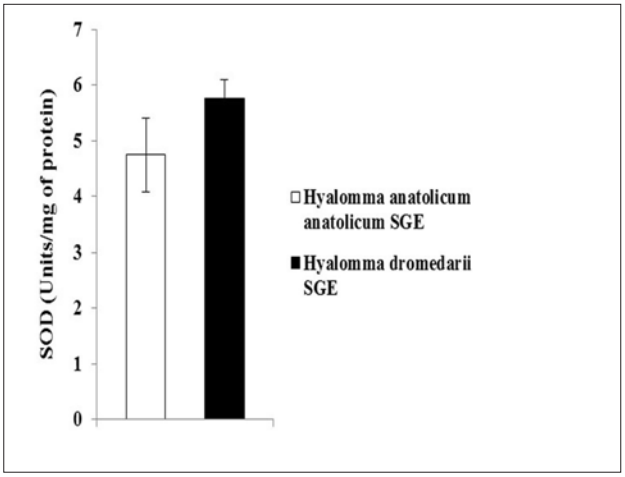

Figure-3: Superoxide dismutase activity (units/mg of protein) in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 Groups, each group containing six ticks.

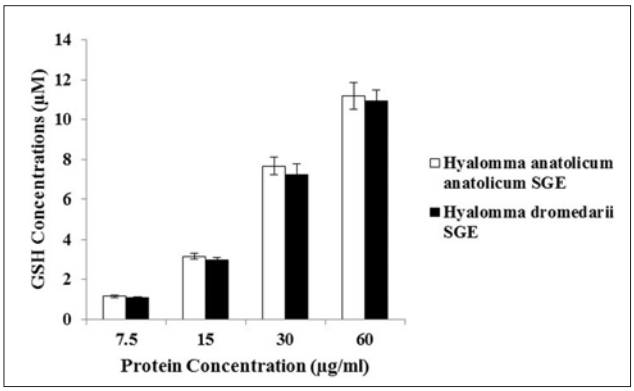

Figure-4: Reduced glutathione concentration in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 Groups, each group containing six ticks.

that ranged from 1.16±0.08 to 11.18±0.66 µM in SGE of *H. a. anatolicum* ticks and from 1.08±0.05 to 10.91±0.57 µM in *H. dromedarii* ticks. Significantly higher GSH concentrations and lower depletion were observed in SGE of *H. a. anatolicum* ticks.

LPO assay

Interspecies variations in MDA level in relation to different protein concentrations (3-24 µg/ml) are presented in Figure-5. The MDA concentrations ranged from 3.45±0.16 to 3.73±0.004 µM in SGE of *H. a. anatolicum* ticks and 3.30±0.02-3.69±0.005 µM in *H. dromedarii* ticks but the interspecies variations were not statistically significant.

Anti-inflammatory activity

Variations in anti-inflammatory activities between the two tick species in relation to different protein concentrations (2.5-20 µg/ml) are presented in Figure-6 where the activity ranged from 17.23±0.54 to 26.08±0.64% in SGE of *H. a. anatolicum* ticks and 19.06±0.53 to 27.87±0.59% in *H. dromedarii* ticks.

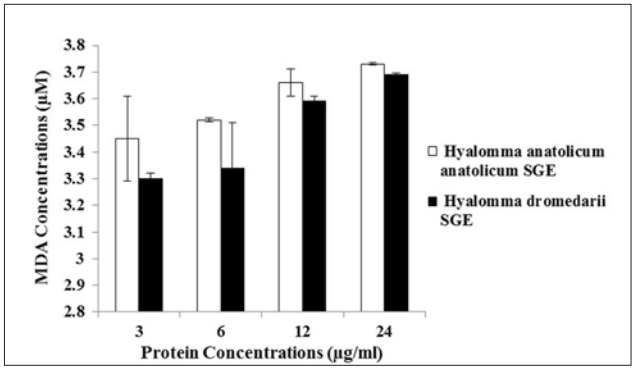

Figure-5: Malondialdehyde concentration in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 Groups, each group containing six ticks.

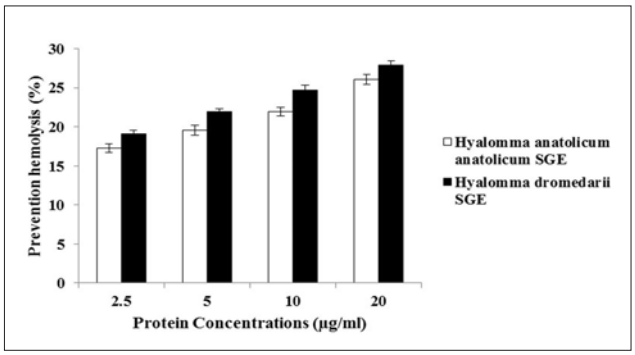

Figure-6: Anti-inflammatory activity in salivary gland extracts of *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* ticks. Data presented as mean±standard error; N=6 Groups, each group containing six ticks.

Significantly higher activities were found in *H. dromedarii* as compared to *H. a. anatolicum* at 2.5 µg/ml, 5.0 µg/ml and 10 µg/ml protein concentrations only.

Discussion

Ixodid ticks due to their prolonged feeding habit on their hosts evokes strong hemostatic, inflammatory and immune responses [4] and results in generation of large amount of superoxides, release of inflammatory mediators, formation of peroxides by activation of lipoxygenases, cyclooxygenase enzymes, release of metal ions from storage sites (iron, copper), generation of free radicals in terms of ROS and RNS, release of hame proteins [2]. Whereas antioxidant response by means of free radical scavenging and detoxification is one of the most important defense mechanism that is employed by the ticks to withstand exogenous and endogenous oxidative stresses during blood feeding. It is crucial to maintain a reducing environment, and this is preserved by redox enzymes that maintain the reduced state through a constant input of many metabolic intermediates by the ticks. Turbulence in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins and lipids (Murray *et al.*, 2003). In present study, higher nitric oxide and hydroxyl radical scavenging activity

in SGE of *H. dromedarii* ticks suggests healthier antioxidant response in these ticks as they are more prone to oxidative stresses during prolonged blood feeding from the host. The interspecies differences observed in the present study were found to be associated with higher engorgement pattern of *H. dromedarii* species than *H. a. anatolicum*. Higher free radical scavenging capacities along with greater SOD activity of *H. dromedarii* ticks might be responsible for making their antioxidant defense further stronger. As hydroxyl radicals (HO·) are the most reactive species of activated oxygen and being a small molecule are highly mobile and are also water soluble. These short-lived molecules which could be produced from O_2 in cell metabolism and under a variety of stress conditions. These HO· radicals once produced have the capacity to damage cells by acting unspecifically on biomolecules located less than a few nanometers from its site of generation [16-19] through redox cycling by Fenton reaction, where a number of redox-active transition - Metals like free iron (Fe2+), reacts with hydrogen peroxide (H_2O_2) and the Haber–Weiss reaction that results in the production of H_2O_2 and Fe2+ when superoxide ($O_2^{\bullet -}$) reacts with ferric iron (Fe3+). Higher SOD activities in *H. dromedarii* in the present study point toward their high catalyzing capacity to dismute $O_2^{\bullet -}$ to O_2 and H_2O_2 [20]. Furthermore, significantly lesser GSH concentrations support the prolonged feeding behavior of these *H. dromedarii* ticks than *H. a. anatolicum* ticks as GSH system is the main H_2O_2 scavenger modulating mitochondrial H_2O_2 emission [21]. Similarly, it was found that proper oxidized/reduced ratios of GSH were maintained by the mitochondrial H_2O_2 buffering through encompassing enzyme activities [21-24]. Hence, the level of GSH depletion could be well-associated with the level of oxidative stress to which the ticks are exposed. Higher GSH depletion in *H. dromedarii* is in accordance with the status of other mentioned components of tick antioxidant defense system in the current work. This could also be supported by the finding of Saeaue *et al.* [25], where they observed depletion in GSH level after repeated blood meal in mosquito. The non-significant variation in MDA concentration in SGE of both the species indicates that they successfully controlled LPO by maintaining MDA level within a safe limit as MDA is widely used as a marker for the peroxidation of ω 3 and ω 6 fatty acids, [26]. MDA is generally believed to originate under stress conditions, and now it has been found to have high capability of reaction with multiple biomolecules such as proteins or DNA that leads to the formation of adducts [27]. It is further reported that excessive MDA production have also been found to be associated with different pathological states [28-31]. Hence, higher anti-inflammatory activity in SGE of *H. dromedarii* ticks might have helped the ticks to feed better by controlling inflammatory response mediated vasoconstriction during tick bite which is also in accordance with increased free radical scavenging activity and overall antioxidant status in these ticks.

Conclusions

In conclusion, higher free radical scavenging activity and antioxidant status with increased GSH consumption in *H. dromedarii* than in *H. a. anatolicum* indicates prominent interspecies variation in antioxidant response according to the feeding behavior and host specificity among the ticks. Anti-inflammatory activity is also in good correlation with antioxidant response. Nonsignificant interspecies variation in MDA level suggests that both the ticks efficiently dealt with the oxidative stress by controlling the LPO.

Authors' Contributions

NS designed the experiment, data analyses and final corrections of the manuscript. MG and AKS collected the sample. AKS identified the tick species and contributed in dissection of ticks. MG performed the dissection of ticks, wet lab analyses, and data analyses. All authors participated in manuscript preparation. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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