Changes in Proteins, Transaminases Activity and Leucocyte Count during *Nerium oleander* Induced Toxicosis in Wistar Rats

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ABSTRACT

This study aimed to find changes in white blood cell profile and liver functions in rats after sterile muscle abscess with *Nerium oleander* leaves decoction and to find out correlation among serum proteins resolved through SDS PAGE. 10ml/kg of the leaves extract injected intramuscularly in Wistar rats (225g, n=4). Control animals were injected with saline injection. All the animals were anesthetized and sacrificed after 3, 6, 12 and 24h of induction. Blood of the control and experimental animals was drawn and processed for analysis. The administration of *N. oleander* induced an acute condition reflected by change in total and differential leucocyte count. Animals in 3, 6 and 24h groups displayed leucocytosis while leucopenia was observed in animals of 12h group. Serum transaminases activity reflected functional changes in liver functions. SDS-PAGE scrutinized variations in serum protein of experimental and control samples and fractions ranged between 52 to 154 KDa. At 6h, 12h and 24h time point, protein fractions of 85KDa or of similar molecular weight were present which seems to be Haptoglobin (86 KDa); a positive acute-phase protein.

Keywords: Acute phase response (APR), Liver functions, *Nerium oleander*, Proteins, SDS-PAGE.

INTRODUCTION

Response to injury generally triggers a spill of cellular reactions that constitute the inflammatory process. The objective of this cellular reaction is to locate and eliminate the byproducts of the lesion (blood and damaged cells) by phagocytosis in preparation for the tissue repair process. Cardinal signs of inflammation are characterized by increased blood flow, elevated cellular metabolism, vasodilatation and release of soluble mediators. Furthermore, variety of the leucocytes is transported at the site of affected tissue to eliminate the causative agent (Kindt et al., 2007; Sheikh et al., 2007; Lundberg & Hansson, 2010). This rapid, sequential and tightly regulated, response accompanied by a number of systemic changes due to injury referred collectively as the acute phase response (APR), which comprised a series of specific physiological reactions including alterations in hematopoietic profile particularly systemic leucocyte mobilization, variety of plasma proteins and serum levels of glucocorticoids and inflammatory cytokines (Ceciliani et al., 2002).

The proteins that respond during APR are referred to as acute phase proteins (APPs) or acute-phase reactants (Furuta et al., 1997; Sheikh et al., 2006). Concentration of APPs is of diagnostic value to therapy in several diseases (Malle & De Beer, 2003). Serum amyloid-A and -P components (SAA and SAP), C-reactive protein (CRP) etc in humans or its homolog in mice are the positive APPs: whose plasma concentration increases while albumin, transferrin, insulin growth factor-1 etc constitutes the negative APPs with decreased plasma concentration (Heinrich et al., 1990; Sheikh et al., 2007).

During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. *Nerium oleander* (*N. oleander*) is one of such worldwide cultivated plants and has been reported with wide range of different therapeutic effects (Yang et al., 2004; Wang et al., 2008; Derwich et al., 2010). Toxic exposure of humans and different species of domestic animals to *N. oleander* cardenolides occurs commonly throughout the geographic regions wherever this plant grows (Hughes et al., 2002; Aslani et al., 2004).

The present study was carried out with the objective to assess the changes in white blood cell profile, transaminases and protein of Wister rat after injection of *N. oleander* aqueous leaves extract during the APR.

MATERIALS AND METHODS

Animals

Male Wistar rats (225g) were kept in a well-ventilated hygienic experimental animal house of Department of Zoology, Govt. College of Science (GCS) (Lahore-Pakistan), under constant environmental and adequate nutritional conditions.

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throughout the period of the experiment. All the animals were acclimatized under standard laboratory condition for a period of 2 weeks prior to the experiment.

**Plant Material and extraction protocol**

Air-dried leaves of *N. oleander* were processed and decoction was made according to the method described by Abbasi et al., 2014. Briefly, leaves were boiled and steam distilled in 0.9 % NaCl solution (1:1, w/v) for 3h. The decoction was filtered and used for the experimental animals.

**Materials**

All chemicals were of analytical grade and obtained from commercial sources as indicated: Kits for the estimation of Plasma amino-transferases and albumin activities from Randox Laboratories, Ltd (U.K). All other reagents and chemicals were from Sigma-Aldrich Chemie (Munich, Germany) and Merck (Darmstadt, Germany).

**Experimental design**

*N. oleander* aqueous leaves extract (10 ml/kg) was administered intramuscularly in both hind limbs using micro-puncture needle (0.25 × 6 mm) to the animals at a volume such that it would permit optimal dosage accuracy without contributing much to the total increase in the body fluid. Control animals received saline injection. The experimental protocol followed a minimally invasive procedure. All the animals were sacrificed at 3, 6 12 and 24h following the extract induction as described elsewhere (Abbasi et al., 2013b).

**Blood Sampling and Processing**

Blood of the control and experimental animals was drawn through direct cardiac puncture and collected in sterilized disposable syringes. 2ml of the blood was transferred to EDTA coated vacutainers (Becton Dickinson, Private Ltd.) for hematological studies and 6-8ml was transferred in the serum gel tubes for further processing and serological analysis.

**Assessment of circulating levels of leucocyte subpopulation**

Complete blood counts were performed for assessment of circulating levels of leucocyte subpopulation in the samples after 6h of collection using an automated blood cell analyzer (Sysmex XT - 1800i, Japan).

**Estimation of Serum Aminotransferases activity**

Blood samples were allowed to clot overnight at 4°C and centrifuged for 20 min at 2000g. Serum was removed and quantitative in vitro determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin activities were determined according to the manufacturer’s instructions.

**SDS-PAGE for evaluation of serum proteins variations**

Laemmli method was used to prepare Polyacrylamide gel (Laemmli, 1970). 8% gel was used to resolve low molecular weight proteins. Briefly, gel plates were set in the electrophoresis chamber and placed in the electrophoresis tank; the gap in the electrophoresis chamber was totally filled with 1x running buffer. The tank was also filled up till the marked level with the buffer. Comb was removed gently in upward direction to keep the wells in its proper shapes. The first well contained 5μl “protein marker” (Fermentas PageRuler™ unstained protein ladder # SM0661) and 15μl of each of the samples were loaded in the successive wells. The voltage was set at 145V and electrophoresis was performed till the tracking dye left the gel and entered the buffer. After the electrophoresis, the two glass plates were separated and was transferred to the staining solution and kept on a shaker for continuous uniform staining of the gel up to 20 minutes following distilled water wash and then immersed into destaining solution on a constant shaking, until the blue colored protein bands became visible against transparent background. (Destaining solution can be changed 2-3 times). After proper destaining, the gel was scanned and images were saved for further analysis.

**Quantification of Protein Fractions**

The densitometry analysis for electrophoretically resolved “protein fractions” was carried out by gel analyzer version 2010a.

**Statistical analysis**

The data were analyzed using Prism Graph pad 5 software (San Diego, CA). Statistical significance was calculated by one-way analysis of variance (ANOVA) and Dunnett post hoc test. Significance was accepted at P < 0.05. Results are shown as Mean ±S.E.M. with n=4.
RESULTS

Total Leucocyte count (TLC) & Differential count

The level of TLC changed significantly during the course of study (P<0.0001). After the onset of APR, time dependent increase was noted with 120.7% increase at 6h and 131% at 24h time point (Fig., 1a).

![Graph showing TLC change](image)

**Fig., 1:** Circulating levels of Total leucocyte count (TLC) and differential count. **a:** TLC, changed significantly during the study with a maximum rise noted after 12h & 24h of injection in comparison with control group (P<0.0001). **b:** Granulocytes; the number of circulating blood neutrophils showed significant increase after 6h and 24h of injection. **c:** Agranulocytes; lymphocytes count showed increase 6h after onset of APR (P<0.001) while statistical significant change was noted for monocyte at all-time points except at 12h as compared to control group. Statistically significant changes are marked with asterisks (*P<0.05, **P<0.01, ***P<0.001; Mean ± S.E.M.; n=4).

Granulocytes count showed marked changes throughout study time points. Statistically significant increase in percentage value was observed in neutrophils at 3.6 & 24h (67.45%, 135.50% & 141.78%, respectively) however decrease was noted at 12h time point when compared with control (P<0.0001). Eosinophil count rose up to 380% at 6h time point however this increase was statistically non-significant as compared to control group (Fig., 1b).

Lymphocyte count showed decrease and then increase at 3h and 6h time points, respectively, while decrease was noted on later time points when compared to control. Monocyte count expressed a statistically significant rise of approximately 413% & 753%, at 3h and 6h after onset of APR while decrease and then increase was noted after 12h and 24h time points, respectively (P<0.0001; Fig., 1c).

![Graph showing differential count](image)

**SERUM ANALYSIS**

Transaminases

A statistically significant increase was observed for AST for all time points compared to control. A rise of 141% in AST at 3h following a steady decrease in the percentage value was noted at later time points. The values noted after 3h were still higher as compared to the control. Serum ALT activity showed 135%, 123.57%, 184.53% and 129.2% rise at 3h, 6h, 12h and 24h time points, respectively (P<0.0001; Fig., 2a). Ratio of transaminases (AST/ALT) was quite high and the
maximum value (1.86) was noted 6h after onset of APR (Fig., 2b).

**SDS PAGE analysis**

The comparative analysis of the serum profile of experimental groups against protein ladder (M) of range 10-220 KDa revealed relative protein fractions from 52 KDa to 154 KDa. The protein fractions of 154 KDa, 84 KDa, 52 KDa, were found 3h after onset of APR compared to control (172 KDa, 150 KDa and 82 KDa). However, protein fractions of 85 and 52KDa were present at 6h time point (Fig., 3b-c). The serum protein fractions of 12h only showed 83 KDa while at 24h time point two bands of 83KDa and 63KDa were resolved compared to protein marker and control (Fig., 3d-e).
Fig. 3: One-dimensional SDS-PAGE and Densitometric comparison of electrophoretically resolved serum proteins of N. oleander leaves decoction treated rat after 3 (b), 6 (c), 12 (d) and 24h (e) time course with sterile muscle abscess compared with control (a) and protein ladder (M).

DISCUSSION

In the current study, effects of intramuscular injection of N. oleander leaves decoction were reported on white blood cell profile, transaminases, and serum proteins of Wistar rats during acute phase reaction.

In the present study, leucocytosis at 3h, 6h and 24h while leucopenia at 12h time point was observed. This might be due to inflammation and tissue necrosis. Leucocytosis, occurs most often due to certain infections or inflammatory processes (Wanahita et al., 2002). This incidence may also be a suggestive of proliferative response by the immune cells followed by immunosuppression at 12h. Abrupt onset of leucopenia could signify margination of neutrophils and their egress into tissues, a process that is sufficiently rapid so as to precede the leucocytosis expected from increased production and release of granulocytes from the marrow storage pool (Theodore E.W., 2007). Physical and emotional stress can also elevate
white blood cell count (Abramson & Melton, 2000; Olipitz et al., 2004). Marked leucocytosis has been reported with 0.1 mg/ml of N. oleander leaves extract/gram of body weight administered orally into albino male mice except for increase in RBC and Hb contents (Narayane et al., 2009). Igwelu and I. A. Fatima, S., Iqbal et al., (2007) reported an increase and then decrease of total leucocyte count and absolute lymphocyte count at the low and subsequent high dose of crude oil administration in male rats.

In the present study, significant neutrophilia occurred except at 12h time point compared with control while the lymphocyte counts showed a decline except at 6h after onset of APR compared to control. Neutrophilia observed might be due to rapid release of young cells from the bone marrow (Doi et al., 1991). These granulocytes undergo a process called chemotaxis, which allows them to migrate toward sites of infection or inflammation (Hubner et al., 1996; Singer & Clark, 1999). Sheikh et al., (2006) reported significant leucocytosis, neutrophilia and decrease of circulating lymphocytes, after subcutaneous and intramuscular turpentine oil injection in rats. Similar changes in the blood cell indices were reported after thioacetamide (TAA) induced acute condition in rats (Abbasi et al., 2013a).

ALT and AST are the members of transaminase family of enzymes and are usually considered as potential marker of liver injury and inflammation. ALT produced mainly in liver and in small amount in other tissues including heart, muscle and kidney while AST is found in various body organs including heart, muscle, kidney, brain and lungs. Rise in the activities of these enzymes is roughly proportional to the extent of tissue damage (Friedman, 1993). Significant increase in AST was observed compared to control with a maximum of 141%. 132.0±11.5 was noted 3h after onset of APR. An increase in serum ALT activities was observed at all-time points when compared to the control. These deranged values reflect the major functional changes in liver functions. Higher level of AST in this study might be due to non-hepatic reasons and might have resulted due to skeletal muscle distortion as in the present study with sterile muscle abscess. N. oleander significantly affected these parameters in a time dependent manner. We found statistically significant elevated levels of both transaminases in the sera at all-time points compared with the control with high AST/ ALT ratio, which indicate the existence of severe inflammation.

The comparative study of the serum protein profile revealed fractions of 52 to 154 KDa and elucidated a considerable variation in the concentration among serum protein. The protein fractions of 154 KDa, 84 KDa and 52 KDa were found 3h after onset of APR compared to control (172 KDa, 150 KDa and 82 KDa). However, at 6h, 12h and 24h time point, protein fractions of 85KD and or of similar density were present. Heptoglobin is a positive acute-phase protein of 86 KDa whose plasma level elevates during inflammation, infections, trauma and malignant proliferation which is evident in the study. This might be due to the sterile inflammation. Elevated protein level in plasma might link with increased risk of multiple diseases, including ischemic heart disease (Kamath & Lip, 2003).

Conclusion

Concluding from the results obtained, it is reported that N. oleander leaves decoction resulted in the onset of acute phase response in rats and its administration leads to changes in the white blood cells total and differential sub populations along with alterations in liver functions with derailed values of transaminases, in time-dependent manner. For the safety, evaluation of the plant extracts is indispensable in order to consider them safe for any treatment.

REFERENCES


