EFFECT OF POMEGRANATE (Punica granatum L) JUICE ON CHANGES IN TISSUE GLUTATHIONE LEVELS OF RATS EXPOSED TOHIGH ALTITUDE HYPOXIA

GAURAVA, PRAVEEN VATS, RADHEY SHYAM, SHOBA SURI MML KUMRIA, K SRIDHARAN, PC SHARMAA, AND SOM NATH SINGH

Department of Biotechnology, Chaudhary charan Singh University, Meerut – 250 004 and Nutrition Division, Defence Institute of Physiology and Allied Sciences, Timarpur, Delhi-110054

Received: 3/11/2000 **Accepted: 12/6/2001**

Abstract: *Oxidative stress due to excessive production of free radicals in living organisms during exposure to hypobaric hypoxia is well documented. In search of a suitable antioxidant from natural sources, in the present study effect of pomegranate (Punica granatum, family Punicaceae) juice (PG) was evaluated on glutathione levels and related enzymes in tissues of rats exposed to simulated altitude of 6096 m. Twenty four male Sprague Dawley rats were divided in three groups i.e. 1) Normal, 2) Exposed to hypoxia and 3) Exposed to hypoxia and treated prior with PG (0.1g/rat) for 15 days. Blood glucose, liver glycogen, glutathione (reduced, GSH; oxidized, GSSG), glutathione reductase, glutathione S-transferase, Y-glutamyl transpeptidase were estimated in liver, muscle and blood/RBC. Marked alterations were observed in these variables during hypoxia exposure. There was decrease in lipid peroxidation in muscle and restoration of GSH:GSSG ratio in PG treated group in comparison with untreated exposed animals. Results confirm recently reported antioxidant property of pomegranate.*

INTRODUCTION:

Various metabolic changes occur in low landers during acclimatization to environmental extremes such as hypobaric hypoxia, cold and increased solar radiation at high altitudes (terrestrial heights more than 3000 meters). There is growing evidence that oxidative stress is more at high altitudes (HA). Increased level of peroxidation markers like thiobarbituric acid reactive substances, hydroxynonenal, 8 hydroxyguanosine in plasma, urine and breath pentane are reported at high altitude. [1-6] Excessive free radical production may contribute to a number of chronic diseases i.e. cancer, lung disease, heart disease and rheumatoid arthritis, [7-10] Dietary supplementation with antioxidant vitamins and minerals (e.g. vitamin E and C,B carotene, selenium and zinc) that act directly as an antioxidant themselves or as co-factor for antioxidant enzymes, may reduce reactive oxygen species (ROS) generation by stimulating antioxidant defence system[4]. Antioxidant supplementation by natural dietary means is an important area of research. Pomegranate (PG) juice (Punica granatum Lin, family Punicaceae) Have emereged as candidate natural product with an antioxidant activity in two recent studies. [11,12] Pomegranate is a well known medicinal plant in Ayurveda and Unani literature. The plant is supposed to be native of Iran and is extensively cultivated as fruit tree or ornamental or for medicinal purposes in

tropical, sub tropical countries such as Spain, Morocco, Egypt, Afganisthan, Iran, India and Far East. Hypoglycemic, anthelmintic, antidysentric activities are well reported. [13-14] Earlier studies from our institute have indicated that during exposure to hypobaric hypoxia, glutathione levels get depleted in rat tissue as well as in human blood. [15-16] Glutathione a tripeptide, γ-glutamyl cysteinyl glycine, occurs in thiol reduced (GSH) and disulfide oxidised form (GSSG) and serves several vital functions including detoxification of electrophiles, scavenging of free radicals, DNA synthesis, microtubular related processes and immune function [17-19] in search of a protective agent against ROS in present study we have evaluated effect of pomegranate juice on glutathione and related enzymic activities in tissues of rats exposed to simulated high altitude 6096 meters. Effect on body weight, blood glucose and glycogen levels were also estimated.

MATERIALS & METHODS

Plant material and preparation of extract/juice: Ripen fruits of Punica granatum were purchased from local market. Epicarp was removed and seeds were separated. Seeds were grinded in mixture grinder and 10% (w/v) extract (juice) was prepared. Juice was filtered through cheese cloth and was frozen in aliquots for further use.

Experimental animals. PG treatment and hypoxia exposure: The Sprague Dawley rats weighing 250 ± 50 g were used in present study. Animals were maintained at 22 ± 2°C with 12h light and dark cycle, fed on standard pellet diet and water ad libitum. Animals were divided into three groups of eight animals in each group1 was kept as normal control and group 3 was treated with pomegranate juice 1 ml (0.1g/rat) per day for 15 days orally. After 15 days group 2 and 3 were exposed to simulated high altitude of 20,000 feet (6,096), pressure equivalent to 349.2 mm Hg. For 72 hrs. Altitude was attained in 20 min at uniform ascent at the rate of 1000 feet/min. Temperature was maintained at 22±2°C at 60% relative humidity. Animals were brought to sea level altitude for 2 hours in evening for change, food and water and body weight change, food intake were recorded. Animals were drawn from heart, liver and thigh muscle were removed, washed in chilled normal saline (0.89% NaCI) and were processed for enzymatic and chemical estimations.

Biochemical estimations: For estimation of glutathione (GSH/GSSG), 50 µl blood was meta phosphoric acid (MPA) and small weighted portions of liver and muscle were homogenized in MPA and 10% w/v homogenate were prepared. GSH and GSSG in acid extracts were estimated fluorometrically by method of Hissin and Hilf,[20] similarly for glycogen estimation tissues were dissolved in 30% potassium hydroxide (w/v), isolation and assay was carried out by method of Montgomery.[21] Rest of the tissue portions were homogenized in 10 volumes of 150 mM KCI. Lipid peroxidation in crude tissue homogenate was estimated as 2-thio barbituric acid reactive substances (TBARS).[22] Crude homogenates were centrifuges at 3,000 gx 15 min. at 4°C and cell free supernatant were divided into aliquots and frozen at -20°C for estimation of enzymes. Erythrocytes were recovered by centrifugation of blood at low speed 1,000 g X 10 min at 4oC, washed twice with phosphate buffered saline pH-[7,4] and lysates (10% w/v in 50mMKCI) were prepared fro enzymic studies. Activity of glutathione rductase (EC 1.6,4.2), glutathione S-transferase (EC 2.5,1.18), γ- glutamyl

transepeptidase (EC 2.3,2.2) were estimated using standard techniques. 23-25 Blood glucose was estimated using method of Nelson as described by Aswell [26]. Protein content of samples were estimated by method of Lowry et al [27.]

Statistical analysis: Data was analyzed using unpaired 't' Test comparision for significance were made between normal and hypoxia exposed groups and hypoxia exposed vs PG treated group and p value <o.o5 was considered significant.

RESULTS

Effect of PG treatment on food intake and body weight is reflected in Fig.1. Animals exposed to simulated HA showed significant reduction (73.2%) in food intake in comparision with normal unexposed rats. PG treatment 15 days prior to exposure and during exposure could not prevent anorexia. Following decrease in food intake there was loss of body weight. There was not much change in liver GSH level, however GSSG level increased significantly (48.4%, $p<0.001$) in HA exposed rats in comparison with normal (Table 1). The PG treatment group also show increase in GSSG levels but when compared with untreated exposed group both GSH and GSSG levels but when compared with untreated HA exposed group GSH and GSSG were increased whereas in untreated groups slight decrease in GSH level was also noted though statistically it was not significant. In case of blood significant increase in both GSH and GSSG levels of HA exposed rats was observed and GSH/GSSG ratio was decreased. PG treatment controlled this increase upto some extent (Table 1, Fig.2).

Lipid peroxidation was increased in all tissue of HA exposed group in comparison with normal. PG treatment provide protection against this increase in muscle however in case of blood lipid peroxidation was even more than HA exposed group (Fig.3)

Effect of HA exposure and PG treatment on enzymic activities of glutathione reductase, glutathione S-transferase and g-glutamyl transpetidase are given in Table 2. Glutathione reductase activity was decreased in HA exposed and PG treated group in comparison with normal animals. In case of muscle, activity was decreased by 46.6 and 49.4% respectively in case of HA exposed and PG treated group in comparison with normal. Glutathione S-transferase activity was decreased in muscle and erythrocytes of HA exposed rats, PG treatment could not prevent this decrease. Γ-GT activity was increased in liver, muscle and erythrocytes of HA exposed rats, PG treatment decreased activity except in case of blood whereas further increase was noted.

HA exposure caused slight rise in fasting blood glucose with significant increase in liver glycogen levels. PG treatment normalized blood glucose level. Glycogen levels in PG treated HA exposed rats were higher by 4.3 and 2.3 times respectively in comparison with normal and HA exposed untreated rats. On the other hand in case of muscle glycogen was depleted in PG treated group in comparison to untreated exposed group (Table 3).

DISCUSSION

Weight loss following exposure to simulated HA observed during study is due to decrease in food intake and is in agreement with earlier studies.[28-34] increase in fasting blood glucose and liver glycogen content have been reported. [34-35] Normalization of increased glucose level in PG treated groups were observed and may be due to its

hypoglycemic activity which is reported in flowers [36]. Increased plasma MDA level and expired pentane in sea level residents have been reported[16] in present study increased levels of TBARS were found in liver, muscle and blood indicating marked oxidative stress. Changes in levels of glutathione were studied as this tripeptide had protective role against oxidative damage. Maintenance of GSH/GSSG ratio is important for cell viability when ratio falls below threshold characteristic of cell population cell viability is affected. Measurement of GSSG is considered as a sensitive marker of oxidative stress during hypoxia.[37] GSH levels are maintained by the activity of glutathione reductase which is decreased in muscle and blood and may responsible for higher levels of GSSH. Glutathione S-transferase, which plays main protective role against electrophilic toxic products of lipid peroxidation is also decreased in case of muscle and erythrocytes whereas in case of specific activity is increased as liver is main site of detoxification reactions.

Activity of γ-glutamyl transpeptidase an enzyme responsible for the transfer of γ-moeity to different amino acids during break down of glutathione conjugate is also increased. Over all results indicate sever oxidative stress following72 hours hypobaric exposure. Work at moderate altitude is also reported to increase oxidative stress and antioxidation supplementation like vitamin E.B-carotene and zinc have little or no effect in controlling it.[5-6] Suitable antioxidant therapies to control oxidative stress have already attracted the world wide attention, nontoxic preferably form natural fource will be of great values. In present study we observed antioxidant effect of PG as observed antioxidant effect of PG as indicated by decrease in lipid peroxidation in muscle and restoration of GSH/GSSG ratio of HA exposed rats which is in agreement with earlier studies. Edible part of pomegranate fruit (about 50% of total fruit weight) comprises about 80% juice and 20% seeds. Fresh juice contains 85% water, 10% sugar and 1.5% pectin, ascorbic acid and polyphenolic flavanoids, Pomegranate seeds are rich source of cruse fibers, pectins and sugar. In pomegranate juice fructose and glucose are present in similar quantities, calcium is 50% of its ash content and principal amino acids are glutamic and aspartic acids.[14-38] The soluble polyphenol contents are 0.2-1.0 % depending on variety and mainly contain catechin, ellagic tannins, cyaniding 3-gllucoside, cyaniding-3-4-diglucoside, gallic and ellagic acids. Fermented pomegranate juice and cold pressed pomegranate seeds possess antioxidant activity and reduce prostaglandin and leukotriene formation by inhibition of cyclooxygenase and lipoxygenase.[11] Recent studies by Aviram et all 12 on pomegninate juice have shown that its consumption reduces oxidative stress in humans as well as atherosclerotic apolipoprotein E-deficient mice. We have supplemented pomegranate juice for 15 days prior and during hypoxic exposure (when oxidative stress is maximum) this may not be sufficient duration. Studies with longer duration in human subjects at high altitude by analyzing blood and urine parameters of oxidative stress may be helpful.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. P.K. Banerjee Sc ‘F’ and Dr, W.Selvamurthy Director, DIP AS for their constant interest and encouragement throughout the study.

REFERENCES

1. Simon-Schnass, 1. Nutrition at high altitude, j Nutr 122, 778-781,(1992).

2. Simon-schnass, 1. Rick of oxidative stress during exercise at high altitude. In: Exercise and Oxygen Toxicity. (Senck, PackerL, Haiminen O, eds,) Elsevier Science BV, New York pp 191-210, (1994).

3. Squires, R.W., Buskirk. E.R. Aerobic capacity during acute exposure to simulated altitude 914-2286 meters, Med, Sci. Sports Excer., 14,36-40, (1982).

4. Askew, E.W. Environmental and physical stress and nutritional requirements, AM. J. Clin Nutr 67 (Suppl), 63IS-637S, (1995).

5. Pfeiffer, J.M., Askew, E.W., Roberts, D.E., Wood, S.M., Benson, J.E., Johnson, S.C., Freedman, M.S. Effect of antioxidant supplementation on urine and blood marker of oxidative stress during extended moderate altitude training, Wilderness Environ. Med., 10,66-74, (1999).

6. Chao, W., Askew, E.W., Roberts, D.E., Wood, S.M. Perkins, J.B. Oxidative stress in humans during work at moderate altitude, J.Nutr., 129,2009-2012, (1999).

7. Kanter, M.M. Free radicals, exercise and antioxidant supplementation. Int J.Sport Nutr., 4,205-220, (1994).

8. Clarkson, P.Antioxidants and physical performance, critical reviews on food science and Nutrition, 35, 131-141, (1995).

9. Packer, L. Oxidants, antioxidants nutrients and the athlete. JSports Science, 15,353-363, (1997).

10. Bandhopadhyay, U., Das D., Banerjee, R.K. Reactive oxygen species; oxidative damage and pathogenesis, current science, 77, 658-566 (1999).

11. Schubert, S. Lanskey, E.P., Neeman, I, Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil fermented juice flavonoids, journal of ethopharmacology, 66,11-17,(1999).

12. Aviram, M. Dornfield, L., Rosenblat, M., Volkova, N., Kaplan,M., Fuhrman, B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice, American Jounal of Clinical Nutrition, 71, 1062-1076, (2000).

13. Satyavati, G.V., Gupta, A.K., Tandon, N.Medicinal plants of India, Vol2, Indian Council of Medical Research, New Delhi, pp-539-544, (1978).

14. Ross, I.A. punica granatum L-Medicinal plants of the World, Humana Press, New Jersey, pp 273-181, (1999).

15. Sairam, M., Sharma, S.K., Dipti, P., Pauline, T., Kain, A.K. Mongia, SS., Bansal, A., Patra, B.D. Illavazhagan, G., Devendra, K., Selvamurthy, W. Effect of hypobaric hypoxia on immunefunction in albino rats, Int. j. Biometerol., 42,55-59, (1998).

16. Illavazhagan, G., Sridharan, K., Sharma, S.K., Bansal, A., Prasad, D., Tomas, P., Kain, A.K. Singh, R., Mongia, S.S., Joshi, G.C., Singh, K., Purkayastha, S.S., Mukerjee, A.K., Kumria, M.M.L., Satija, N.K., Sharma, R.P., Vats P., Chand, T., Kumar, D., Selvamurthy, W. Role of Vitamin C and E under high altitude combat stress, Defence Institute of Physiology and Allied Sciences, Report No. DIPAS/26/96, (1996).

17. De Leve, L., Kaplowitz, N. Glutatione metabolism and its role in hepatotoxicity, Pharmacol, Ther., 52, 287-305, (1991).

18. Meister, A., Anderson, M.E. Glutathione metabolism and its role in hepatotoxicity, Ann. Rev. Biochem., 52,711-760, (1983).

19. Lu, S.C. Regulation of hepatic glutathione synthesis: current concepts and controversies,. Current concepts and controversies,. FASEB j., 13, 1169-1183, (1999).

20. Hissin, P.J., Hilf, R.A fluourometric method of oxidized and reduced glutathione in tissues, Anal. Biochem., 74,214-226,(1976).

21. Montgomery, R.Determination of glycogen, Arch. Biochem. Biophys., 67,378-386, (1957).

22. Utley, H.G., Bemheim, F., Hochstein, P. Effect of sulfhydryl reagents on peroxidation of microsomes, Arch. Biochem. Biohys., 118, 29-32, (1967).

23. Bemji, M.S. Glutathione reductase activity in red blood cells and riboflavin nutritional status in human, Clin, Chem. Acta., 26,263-269, (1969).

24. Habig, H.W., Jakoby, W.B. Assay for transferases, Methods Enzymol., 77,398-405, (1981).

25. Orlowsky, M. and Meister, A. γ-Glutamyl p-nitroanilide, a new convenient substrate for determination and study of L and D- γ-glutamyl transpeptidases activities, Biochim. Biophys., Acta., 73,679-681, (1963).

26. Aswell, G. Colorimetric analysis of sugars, Methods in Enzymology, Vol III, 85-86 (1957).

27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the folin phenol reagent, J.Biol. Chem., 193,265-275, (1951).

28. Surks, M.L., Chinn, K.S.K., Matouch, LO. Alterations in body composition in man after acute exposure to high altitude, journal of applied physiology, 21,1741-1745, (1966).

29. Boyers, S.J., F.D. Weight loss and changes in body composition at high altitude journal of applied physiology, 57, 1580-1585, (1984).

30. Rose, M.S., Houston, C.S., Fulco, C.S., Coates, G, Carlson, D., Sutton, J.R., Cymerman, A. Operation Everest II-Nutrition and body composition, journal of applied Physiology, 65,2545-2551, (1988).

31. Sridharan, K., Mukherjee, A.K., Grover, S.K., Kumria M.M.L, Arora, BS., Rai, R.M. Assessment of nutritional status and physical work capacity of toad construction workers at altitude of 2 150-2750 m on two different ration scales, Nutrition ReportsInternational,35, 1269-1277, (1987).

32. Butterfield, G.E., Gates, J., Fleming, S., Brook, G.A., J.R., Reeves, J.T. Increased energy intake minimizes weight loss in men at high attitude, J. Appl. Physiol., 72,1741-1748, (1992).

33. Singh, S.B., Sharma, A., Sharma, K.N., Selvamurthy, W. Effect of high altitude hypoxia on feeding responses and hedonic matrix in rats. J Appl. Physiol., 80, 1133-1137, (1996).

34. Vats, P., Mukherjee, A.K., Kumria, M.M.L., Singh, S.N., Patil, S.K.B., Ranganathan, S., Sridharan, K. Changes inactivity levels of glutamine synthetase, glutaminase and glycogen synthetase in rats subjected to hypoxic stress, Int.J. Biometereol., 42,205-209, (1999).

35. Van Liere, E.J., Stickney, J.C. Chemical changes in the blood during hypoxia In: Hypoxia, The University of Chicago Press, Chicago pp 61-75, (1963).

36. Jafri, M.A. Effect of Punica granatum Linn. (flowers) on blood glucose level in normal and alloxan induced diabetic rats, J. Ethnopharmacol., 70 (3), 309-314, (2000).

37. Jae Schke, H. Glutathione disulfide as index of oxidative stress in rat liver during hypoxia, Am. J. Physiology, 258,G 499-G 505, (1990).

38. Rastogi, R.P., Mehrotra, B.N. Compendium of Indian Medicinal plants, Vol 2, CDRI, Lucknow and PID, New Delhi, pp 573, (1993).

Table 1: Reduced and oxidized Glutathione levels in normal, high altitude exposed and treated rats

GROUPS	GSH LIVER (µmol/g wet tissue)	MUSCLE	BLOOD (µmol/ml)	LIVER (µmol/g wet tissue)	GSSG MUSCLE	BLOOD (µmol/ml)
NORMAL	4.67 ± 0.21	0.62 ± 0.04	1.10 ± 0.02 ++	0.64 ± 0.30++	0.14 ± 0.006	0.071 ± 0.003 ++
HA EXPOSED	5.51 ± 0.37	0.51 ± 0.08	1.69 ± 0.04	0.95 ± 0.05 +,*	0.16 ± 0.011 ++	0.176 ± 0.007 ++,*

			++,**			
HA EXP-	5.35 ± 0.35	0.71 ± 0.07	1.36 ± 0.05	0.77 ± 0.03	0.17 ± 0.005	0.125 ± 0.006

Values are Mean-SEM,n =8
=p<0.01, ++p<0.001 in comparison with normal
*p<0.01, **p<0.001 in comparison with HA Exposed

Table 2: Effect of HA exposure and PG treatment on enzymic activities of glutathione reductase, glutathione S-transferase and y-glutamyl transpeptidase

GROUP	GR[a]			GST[a]			y-GTc[a]		
	LIVER	RBC	MUSCLE	LIVER	RBC	MUSCLE	LIVER	RBC	MUSCLE
NORMAL	48.52 ± 8.34	2.68 ± 0.23	12.51 ± 0.21	541 ± 90	20.4 ± 2.1	59.5 ± 4.0	2.58 ± 0.34	0.43 ± 0.09	1.31 ± 0.20
HA EXPOSED	42.22 ± 4.71	2.24 ± 0.30	6.67 +++ ± 0.46	704 ± 27	13.9 ± 1.7	31.2+++ 2.0±	7.16 ± 0.69	0.73+ ± 0.08	1.65 ± 0
HA EXPOSED & PG TREATED	34.36 * ± 2.60	1.24+++ ± 0.10	6.33 +++ ± 0.43	597 ± 46	92 +++NS ± 0.4	30.1 +++NS 2.0±	5.05 +++,* ± 0.27	0.90++ ± 0.09	1.29 ± 0.16

Values are Mean-SENM (n=8)
a-nmol NADPH oxidized/min/mg protein, b-nmol thioester formed/min/mg protein
c-nmol p-nitroaniline released /min/mg protein
p<0.05,++p<0.0001 in comparison with normal
*p<0.05 in comparison with HA exposed
NS-No significant change in HA exposed and PG treated.

Table 3: Effect of HA Exposure and PG treatment on blood glucose and glycogen levels in liver and muscle

GROUP	BLOOD GLUCOSE (mg/dl)	GLYCOGEN (mg/g wet tissue)	
		LIVER	MASCLE
NORMAL	74.12 ±2.58	4.62 ± 0.71	4.46 ± 0.41
HA EXPOSED	86.08 ±3.69	8.80 ± 0.92	4.52 ± 0.26
HA EXPOSED TREATED	76.20 ± 6.15	20.04 ± 2.41	2.94 ± 0.30

Values are Mean SEM, n=8
+p<0.05, ++p<0.005,+++p<0.001 in comparison with normal
*p<0.0005. **p<0.001 in comparison with HA exposed

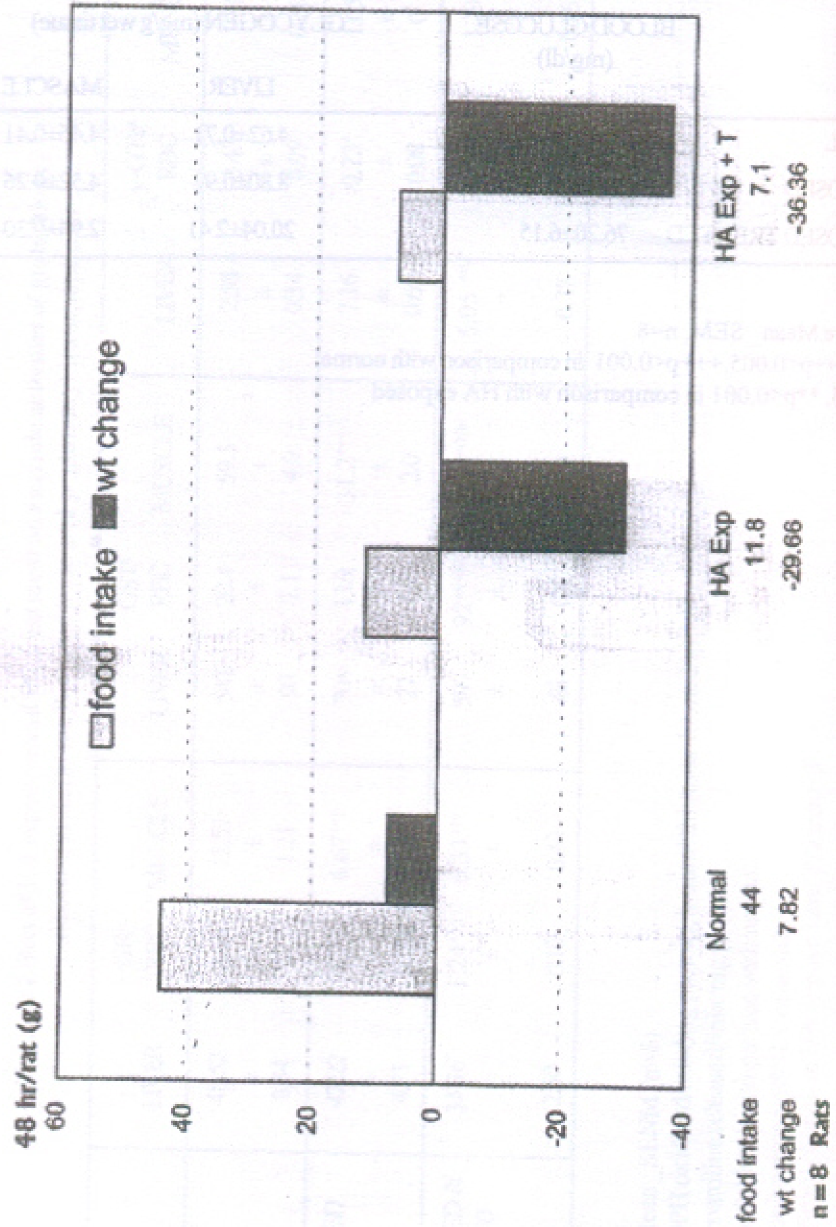


Fig: 1

84

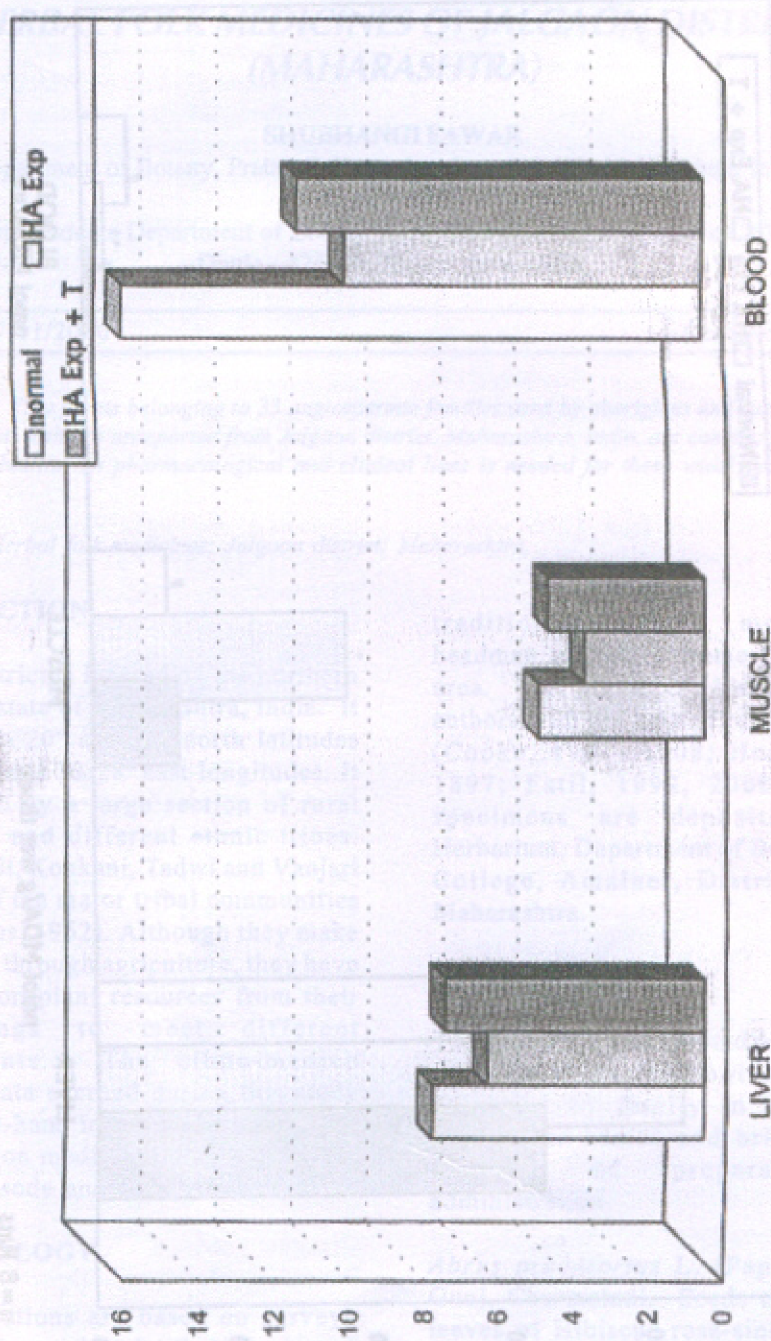
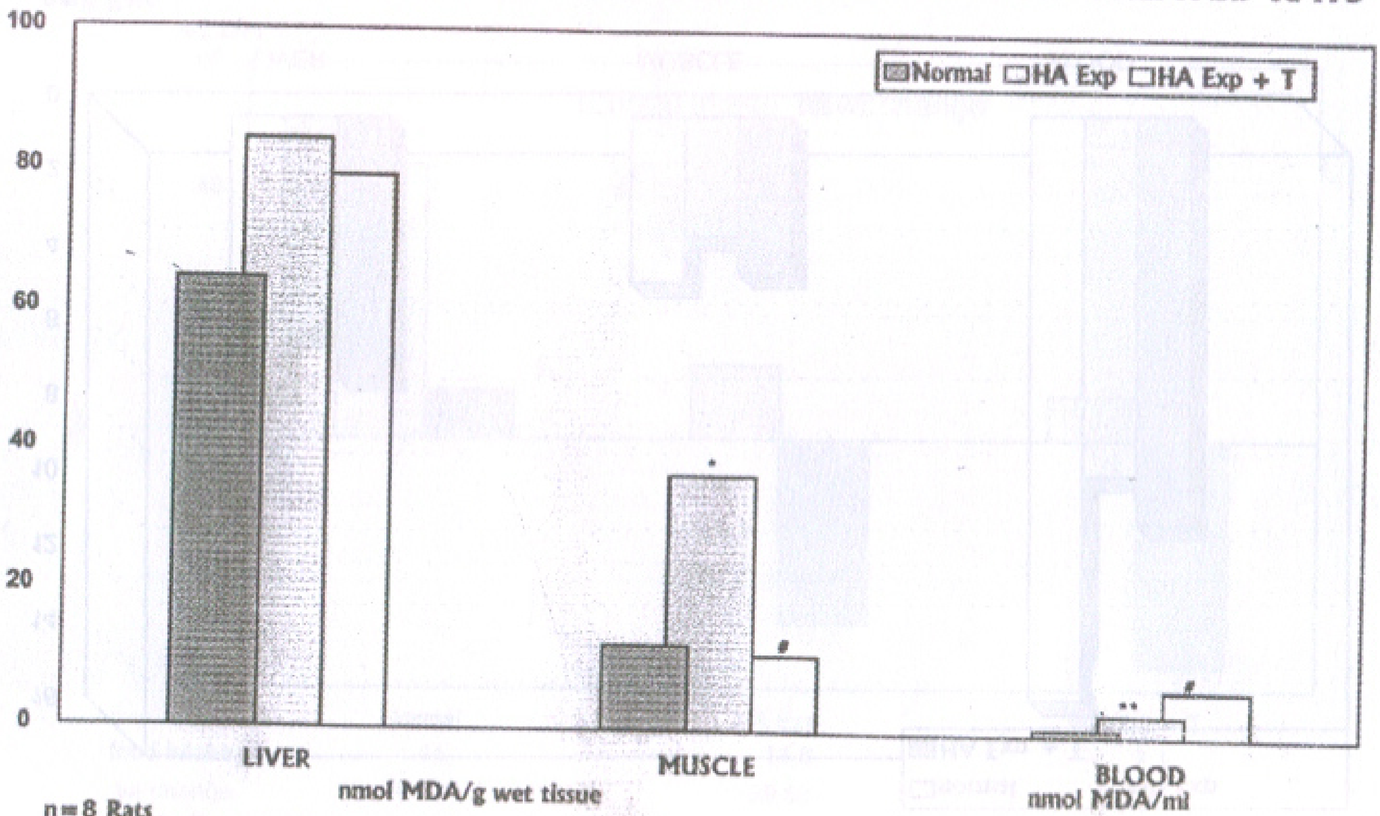


Fig: 2



n=8 Rats
* $p<0.05$, ** $p<0.001$ in comparison with normal, # $p<0.05$ in comparison with HA Exp

Fig: 3

86