# EFFECT OF POMEGRANATE (*Punica granatum*) JUICE ON PLASMA NITRIC OXIDE LEVELS AND ANTIOXIDANT STATUS IN ISOLATION STRESS AND HEAT STRESS INDUCED RATS

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## ABSTRACT

*This study was undertaken to assess the effect of pomegranate (Punica granatum) juice (PJ) supplementation on plasma nitric oxide (NO) levels and antioxidant status in isolation stress induced and heat stress induced rats. Thirty adult male Wistar albino rats of 140-150 g body weight were randomly divided into five groups of six each viz., control, isolated stress induced, isolation stress induced supplemented with PJ, heat stress induced and heat stress induced supplemented with PJ. Blood samples were collected at weekly interval during four weeks of the experimental period, plasma samples were separated and stored at -20$^0$C for the assay of nitric oxide (NO) levels, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) activities and lipid peroxidation levels. PJ supplementation to isolated stress induced and heat stress induced rats significantly (Pd" 0.05) increased plasma nitric oxide (NO) levels, SOD, CAT and GSH-Px activities, whereas plasma lipid peroxidation level was significantly lowered (Pd" 0.05) in comparison with respective stress induced models and control groups.*

**Key words:** Pomegranate juice (PJ), Isolation stress and heat stress induced – Nitric oxide (NO) - SOD, CAT and GSH-Px, lipid peroxidation – Rats.

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## INTRODUCTION

Stress is a state of threatened homeostasis provoked by psychological, physiological or environmental stressors. An animal reared under extreme confinement may be physically, socially and psychologically stressed; of which psychological stress is the most potent. Oxidative stress occurs when homeostasis is tipped towards an overbalance of free radicals, due to either overproduction of reactive oxygen species (ROS) or deficiency of antioxidant defense in during stress (Sies, 1993).

Exposure to stressful stimuli has been found to induce the activation of NO producing neurons in the amygdala and hypothlamus (Krukoff and Khalili, 1997). Nitric oxide, the abundant reactive radical acted as an important oxidative biological signaling molecule in large variety of diverse physiological processes, including neurotransmission, blood pressure regulation. Defense mechanism, smooth muscle relaxation and immune regulation (Bergendi *et al.,* 1999).

NO modulated the hypothalamic-pituitary-adrenal axis (Bilbo *et al.,* 2001). Increased expression of nNOS (Oliviera *et al.,* 2002) and iNOS (Madrigal *et al.,* 2001, 2003; Homayoun *et al.* 2002) occurred in limbic brain region following acute restraint stress in rats. Esch *et al.* (2002) stated that nitric oxide (NO), a stable gaseous free radical, played a role in many stress related diseases.

Kavita *et al.* (2006) reported that, NO played an important regulatory role in the susceptibility and adaptation to stress. NO played a crucial modulatory role in stress induced neurobehavioural effects (Khan and Ghosh, 2010).

Oxidative stress occurs when homeostasis is tipped towards an overbalance of free radicals, due to either overproduction of Reactive oxygen species (ROS) or deficiency of antioxidant defense in favour of stress (Sies, 1993). The most common ROS include superoxide anion, hydrogen peroxide ($H_2O_2$), peroxyl ($ROO^-$) radicals and reactive hydroxyl ($OH^{-)}$) radicals.

Superoxide dismutase, the first line of defense against the deleterious effect of oxygen radicals in the cells, scavenges ROS by catalyzing the dismutation of superoxide to $H_2O_{2.}$ The utilization of SOD activity might result in an increased flux of superoxide in the cell which might be the reason for increased lipid peroxidation (Devipriya *et al.,* 2007). Devipriya *et al.* (2007) stated that oxidative stress significantly decreased catalase activity in rats under stress or normal condition. Turk *et al.* (2008) reported that the different doses of pomegranate juice showed marked increase in GSH-Px and CAT activities in rats. PJ consumption resulted in reduction of lipid peroxides in human beings (Aviram *et al.,* 2004). Different doses of PJ significantly decreased plasma MDA levels (Turk *et al.,* 2008). Pomegranate juice supplementation to

LDLR mice under oxidative stress substantially lowered plasma lipid peroxidation (Nigris *et al.,* 2007). Pomegranate juice consumption reduced plasma lipid peroxide concentration in mice (Kaplan *et al.,* 2009).

## MATERIALS AND METHODS

Thirty adult male Wistar albino rats of 140 -150 g body weight were randomly divided into five groups of six each viz., control, isolated stress induced, isolation stress induced supplemented with PJ, heat stress induced and heat stress induced supplemented with PJ were reared at Centralized Laboratory Animal house, Madras Veterinary College, Chennai -7 under standard managemental practice. The rats were fed with standard rodent pellet feed and water *ad libitum*. Isolation stress was induced by placing each rat in separate cages throughout the experimental period. Thermal stress was induced by incandescent bulbs of 40 watts at a distance of 30 cm from the floor of the cage for forty five minutes daily from the commencement of the experiment to 28$^{th}$ day of completion of the experiment. PJ was extracted by crushing 100 g of seed and 1ml of undiluted fresh juice (Turk *et al.,* 2008) was given p/o to each rat daily. This experimental trail was approved by Institutional Animal Ethical Committee (Lr. No. 1937/DFBS/IAEC/A/2009 dt. 20.07.2009).

Blood samples were collected from orbital sinus plexus at weekly intervals and plasma samples were separated by centrifugation at 3000 rpm for 15 min at 4$^0$c and stored at -20$^0$C until spectrophotometric analysis of plasma NO levels, SOD, CAT, GSH-Px activities and lipid peroxidation levels were over.

Nitric oxide levels in the plasma were indirectly measured spectrophotometrically using the products of NO namely nitrite ($NO_2$) and nitrate ($NO_3$) as per the method of Guevara *et al.* (1998).

SOD activity in the plasma was measured as per the method of Marklund and Marklund (1974). Catalase activity was determined according to the method of Caliborne (1985). GSH-Px activity was assessed as per the method of Rotruck *et al.* (1973). Lipid peroxidation was estimated according to the method of Yagi (1976).

The parameters were statistically analyzed by completely randomized block design and randomized block design as per the method of Snedecor and Cochran (1989).

## RESULTS AND DISCUSSION

The results of this study on the effect of PJ on plasma NO levels, SOD, CAT, GSH-Px activities and lipid peroxidation levels in isolation stress induced and heat stress induced rats are presented in the Table.

Plasma NO levels ranged between 5.00 ± 1.75 and 8.83 ± 1.18 µm/dl in isolation stress induced rats. In isolation stress induced rats supplemented with PJ, the plasma NO levels ranged between 14.10 ± 4.15 and 23.22 ± 0.73

μm/dl. In heat stress induced rats, plasma NO levels ranged between 10.73 ± 2.07 and 16.40 ± 3.60 μm/dl. Plasma NO levels ranged between 23.27 ± 1.73 and 30.28 ± 5.23 μm/dl in heat stress induced rats supplemented with PJ.

Both isolation stress and heat stress induced rats supplemented with PJ revealed significantly ($P \leq 0.05$) higher NO levels when compared to respective stress induced groups. NO levels in isolation stress induced rats supplemented with PJ were significantly ($P \leq 0.05$) lower than that of control group, whereas NO levels in heat stress induced rats supplemented with PJ and control group did not show significant variation. Similarly there was no significant difference between the periods of treatment.

In restraint stress exposed rats lowered NO activity was responsible for the stress induced anxiogenic effect (Kavita *et al.,* 2006). Similarly in the present study also there was a significant decrease in the plasma NO level. Plasma and brain NO activities were lower in restraint stress exposed rats and indicated that restraint stress effect on corticosterone might be associated with low levels, probably via inhibition of NO synthase (Kavita *et al.,* 2006).

Ignarro *et al.* (2006) stated that PJ augmented the action of NO via their antioxidant activity to protect and maintain the functional levels of NO within smooth muscle cells in culture.

PJ extract displayed a similar range of effects, increasing eNOS expression and NO release in cultured human coronary aortic endothelial cells as well as *in vivo* in the vasculature of diabetic rats (Nigris *et al.,* 2007 a; Nigris *et al.,* 2007 b).

Hence the antioxidant activity of PJ would have protected the NO synthase from oxidative destruction and this might be the reason for significant increase in NO levels in PJ supplemented groups in both stress induced models of the present study.

Plasma SOD activity ranged between 11.14 ± 0.14 and 18.39 ± 0.39 U/min/mg protein in isolation stress induced rats. In isolation stress induced rats supplemented with PJ, the plasma SOD levels ranged between 25.65 ± 0.65 and 41.30 ± 3.33 U/min/mg protein. In heat stress induced rats, plasma SOD activity ranged between 13.07 ± 1.79 and 18.92 ± 0.15 U/min/mg protein. In heat stress induced rats supplemented with PJ, the plasma SOD activities ranged between 19.63 ± 1.37 and 39.49 ± 5.60 U/min/mg protein. Both isolation stress induced and heat stress induced rats supplemented with PJ revealed significantly higher SOD activities as compared to respective stress induced control groups. In isolation stress induced rats, significantly higher ($P \leq 0.05$) SOD activity was recorded in the fourth week of treatment with PJ in comparison with first two weeks of treatments, whereas PJ treatment in heat stress induced rats during fourth week showed no significant

($P\leq 0.05$) difference when compared to control group.

SOD detoxified hydrogen peroxide and converted lipid hydroperoxides to non-toxic alcohols (Guemouri *et al.,* 1991). Okado and Fridovich (2001) also reported that SOD scavenged ROS by catalyzing the dismutation of superoxide to $H_2O_2$.

Pomegranate had free radical scavenging and potent antioxidant activities (Nigris *et al.,* 2005; Balasundram *el. al.,* 2006; Rosenblat *et al.,* 2006). The most abundant polyphenol in pomegranate juice are the hydrolysable tannins called ellagitannins also called as punicalagins. Abundance of ellagitannin in PJ significantly increased SOD against ethanol induced oxidative stress in rats (Devipriya *et al.,* 2007).

Plasma catalase activities ranged between 200.44 ± 19.07 and 220.90 ± 0.79 m.mol/l in isolation stress induced rats. In isolation stress induced rats supplemented with PJ, the plasma catalase activities ranged between 218.13 ± 6.87 and 388.49 ± 3.08 m.mol/l.In heat stress induced rats, plasma catalase activities ranged between 185.71 ± 4.28 and 255.73 ± 11.55 m.mol/l. In heat stress induced rats supplemented with PJ, Plasma catalse activities ranged between 295.84 ± 13.29 and 397.69 ± 5.47m.mol/l. Both isolation stress induced and heat stress induced rats supplemented with PJ revealed significantly higher ($P\leq 0.05$) catalase activities in the third and fourth week of treatment in comparison with the control group.

Turk *et al.* (2008) also reported that PJ supplementation showed dose dependant increase in CAT activities in stress induced rats. Catalse played an important role in the protection against the deleterious effects of LPO (Devipriya *et al.,* 2007).

Plasma GSH-Px activities ranged between 10.70 ± 0.48 and 18.28 ± 0.71 µg/min/mg protein in isolation stress induced rats. In isolation stress induced rats supplemented with PJ, the plasma GSH-Px activities ranged between 43.80 ± 3.45 and 62.50 ± 0.50 µg/min/mg protein. In heat stress induced rats, plasma GSH-Px activities ranged between 23.21 ± 1.81 and 35.28 ± 1.75 µg/min/mg protein. Plasma GSH-Px activities ranged between 95.16 ± 7.02 and 126.00 ± 16.00 µg/min/mg protein in heat stress induced rats supplemented with PJ. Both isolation stress induced and heat stress induced rats supplemented with PJ revealed significantly higher ($P\leq 0.05$) GSH-Px activities when compared to respective stress induced control groups. Isolation stress induced rats supplemented with PJ had significantly lower ($P\leq 0.05$) GSH-Px activities in comparison with control group.

Guemouri *et al.* (1991) stated that GSH-Px detoxified hydrogen peroxides by converting it to non-toxic alcohols.

Plasma lipid peroxidation levels ranged between 66.40 ± 1.20 and 102.70 ± 10.50 nM of MDA/ml in isolation stress induced rats. In isolation stress induced rats supplemented with PJ, the plasma lipid peroxidation levels ranged between 31.20 ± 5.20 and 53.10 ± 0.90 nM of MDA/ml. There was a gradual and significant decrease ($P \leq 0.05$) in the lipid peroxidation levels from first week to fourth week of treatment with PJ. Significantly lower ($P \leq 0.05$) level was observed during fourth week of PJ treatment in comparison with first two weeks of treatment. Isolation stress induced rats supplemented with PJ revealed significantly lower ($P \leq 0.05$) lipid peroxidation when compared to control group.

In heat stress induced rats, plasma lipid peroxidation levels ranged between 123.50 ± 1.30 and 137.99 ± 2.41 Plasma lipid peroxidation levels ranged between 28.25 ± 2.25 and 67.25 ± 1.25 nM of MDA/ml in heat stress induced rats supplemented with PJ. Heat stress induced rats supplemented with PJ revealed significantly lower ($P \leq 0.05$) lipid peroxidation when compared to control and heat stress induced groups. Significantly lower ($P \leq 0.05$) lipid peroxidation was recorded during fourth week of treatment with PJ when compared to other periods.

This could be due to high content of ellagitannins in PJ that acted as a hydrogen ion donor and acceptor which might be involved in free radical scavenging action and decreased free radiacl mediated lipid peroxidation (Aviram *et al*., 2004; Bala *et al.,* 2006; Nigris *et al.,* 2007).

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## Table

**EFFECT OF POMEGRANATE (*Punica granatum*) JUICE ON PLASMA NO LEVELS, ANTIOXIDANT STATUS IN ISOLATION STRESS AND HEAT STRESS INDUCED RATS**

| **Groups** | **Plasma NO levels (µm/dl)** | | | |
|---|---|---|---|---|
| | **1st week** | **2nd week** | **3rd week** | **4th week** |
| Control | $32.50^{aA} \pm 2.50$ | $30.70^{aA} \pm 0.21$ | $37.15^{aA} \pm 1.65$ | $35.50^{aA} \pm 1.00$ |
| Isolation stress induced | $07.85^{bA} \pm 1.60$ | $05.00^{cA} \pm 1.75$ | $08.83^{cA} \pm 1.18$ | $07.60^{cA} \pm 1.50$ |
| Isolation stress induced + PJ | $14.10^{aA} \pm 4.15$ | $16.25^{bA} \pm 3.75$ | $21.43^{bA} \pm 1.43$ | $23.22^{bA} \pm 0.73$ |
| Heat stress induced | $10.73^{bA} \pm 2.07$ | $16.05^{bA} \pm 2.25$ | $16.40^{bA} \pm 3.60$ | $13.00^{bA} \pm 2.12$ |
| Heat stress induced + PJ | $23.27^{aA} \pm 1.73$ | $26.88^{aA} \pm 3.13$ | $30.28^{aA} \pm 5.23$ | $28.38^{aA} \pm 4.88$ |
| **Groups** | **Plasma SOD activities** (U/min/mg protein) | | | |
| | **1st week** | **2nd week** | **3rd week** | **4th week** |
| Control | $39.53^{aA} \pm 0.74$ | $40.39^{aAB} \pm 2.39$ | $45.53^{aB} \pm 0.47$ | $41.68^{aAB} \pm 0.31$ |
| Isolation stress induced | $18.39^{aA} \pm 0.39$ | $14.64^{aA} \pm 3.36$ | $14.28^{aA} \pm 3.00$ | $11.14^{aA} \pm 0.14$ |
| Isolation stress induced + PJ | $25.65^{bA} \pm 0.65$ | $28.19^{bA} \pm 1.89$ | $33.81^{baB} \pm 3.79$ | $41.30^{bB} \pm 3.33$ |
| Heat stress induced | $14.73^{cA} \pm 0.27$ | $16.64^{cA} \pm 2.14$ | $13.07^{cA} \pm 1.79$ | $18.92^{bA} \pm 0.15$ |
| Heat stress induced + PJ | $19.63^{bA} \pm 1.37$ | $28.19^{bA} \pm 1.80$ | $35.67^{bAB} \pm 1.86$ | $39.49^{aB} \pm 5.60$ |

| Groups | Plasma CAT activities (m.mol/l) | | | |
|---|---|---|---|---|
| | 1st week | 2nd week | 3rd week | 4th week |
| Control | 269.24$^{aA}$±5.24 | 282.40$^{aA}$±2.24 | 280.99$^{aA}$±5.99 | 296.69$^{aA}$±3.30 |
| Isolation stress induced | 206.78$^{aA}$±3.21 | 204.00$^{aA}$±1.00 | 220.90$^{aA}$±0.79 | 200.44$^{cA}$±19.07 |
| Isolation stress induced + PJ | 218.13$^{aA}$±6.87 | 251.03$^{bB}$±5.24 | 362.92$^{cC}$±2.07 | 388.49$^{bC}$±3.08 |
| Heat stress induced | 255.73$^{aA}$±11.55 | 237.75$^{aAB}$±2.83 | 210.47$^{aBC}$±10.47 | 185.71$^{cC}$±4.28 |
| Heat stress induced + PJ | 307.50$^{aA}$±55.94 | 295.84$^{bA}$±13.29 | 379.78$^{cA}$±14.50 | 397.69$^{cA}$±5.47 |
| **Groups** | **Plasma GSH-Px activities** (µg/min/mg protein) | | | |
| | 1st week | 2nd week | 3rd week | 4th week |
| Control | 96.28$^{aA}$±10.33 | 103.99$^{aA}$±4.59 | 99.79$^{aA}$±17.40 | 108.00$^{aA}$±2.00 |
| Isolation stress induced | 10.70$^{cA}$±0.48 | 17.81$^{cAB}$±2.48 | 15.17$^{bAB}$±2.40 | 18.28$^{cB}$±0.71 |
| Isolation stress induced + PJ | 43.80$^{bA}$±3.45 | 58.52$^{bA}$±5.10 | 61.74$^{aB}$±4.26 | 62.50$^{bB}$±0.50 |
| Heat stress induced | 35.28$^{bA}$±1.75 | 25.42$^{cAB}$±1.40 | 32.70$^{bAB}$±4.70 | 23.21$^{bA}$±1.81 |
| Heat stress induced + PJ | 95.16$^{aA}$±7.02 | 113.42$^{aA}$±4.42 | 111.94$^{aA}$±21.55 | 126.00$^{aA}$±16.00 |
| **Groups** | **Plasma Lipid peroxidation levels** (nM of MDA/ml) | | | |
| | 1st week | 2nd week | 3rd week | 4th week |
| Control | 98.20$^{aA}$±10.33 | 98.70$^{aA}$±2.70 | 92.00$^{aA}$±4.00 | 92.50$^{aA}$±7.50 |
| Isolation stress induced | 66.40$^{cA}$±1.20 | 92.33$^{aB}$±10.33 | 102.70$^{aB}$±10.50 | 92.10$^{aB}$±1.50 |
| Isolation stress induced + PJ | 53.10$^{bC}$±0.90 | 45.50$^{bBC}$±1.00 | 35.30$^{bAB}$±1.10 | 31.20$^{bA}$±5.20 |
| Heat stress induced | 123.50$^{cA}$±1.30 | 137.99$^{cA}$±2.41 | 137.99$^{cC}$±2.41 | 137.60$^{cC}$±2.25 |
| Heat stress induced + PJ | 67.25$^{bC}$±1.25 | 60.00$^{bAB}$±8.00 | 60.00$^{bBC}$±8.00 | 28.25$^{bA}$±2.25 |