Research Paper

Cardioprotective potential of *Punica granatum* extract in isoproterenol-induced myocardial infarction in Wistar rats

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ABSTRACT

Objective: To determine the protective role of *Punica granatum* L. (Punicaceae) seed juice extract and its butanolic fraction on heart rate, electrocardiographic patterns, vascular reactivity to catecholamines, cardiac marker enzymes, antioxidant enzymes together with morphologic and histopathological changes in isoproterenol-induced myocardial infarction in male Wistar rats. **Materials and Methods:** The effects of *Punica granatum* seed juice extract (100 mg/kg, p.o. and 300 mg/kg, p.o.) and butanolic fraction of *Punica granatum* seed juice extract (100 mg/kg., p.o.) on cardiac parameters were studied. Isoproterenol hydrochloride was used to induce myocardial infarction in Wistar rats. At the end of the experiment, heart rate, ECG, pressure rate index and cardiac marker enzyme levels were assessed. **Results:** Rats treated with isoproterenol (85 mg/kg, administered subcutaneously twice at an interval of 24 h) showed a significant increase in heart rate, ST elevation in ECG, pressure rate index and a significant increase in the levels of cardiac marker enzymes- lactate dehydrogenase, and creatine kinase in serum. Isoproterenol significantly reduced superoxide dismutase and catalase activity and increased vascular reactivity to various catecholamines. Pretreatment with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) for a period of 21 days significantly inhibited the effects of ISO on heart rate, PRI, ECG patterns, levels of LDH, CK, SOD, CAT, and vascular reactivity changes. Treatment with PJ (100 mg/kg and 300 mg/kg) and B-PJ (100 mg/kg., p.o.) alone did not alter any of the parameters as compared to vehicle-treated Wistar rats. *Punica granatum*-treated animals showed a lesser degree of cellular infiltration in histopathological studies. **Conclusion:** *Punica granatum* ameliorates cardiotoxic effects of isoproterenol and may be of value in the treatment of MI.

Key words: Antioxidant enzymes, cardiac marker enzymes, ECG, isoproterenol, *Punica granatum*

INTRODUCTION

An increased production of free radicals or generation of toxic reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical gives rise to oxidative stress, which plays a major role in cardiovascular diseases such as ischemic heart disease, atherosclerosis, congestive heart failure, cardiomyopathy and arrhythmias.[1] Isoproterenol {4-[1-hydroxy-2-(1-methylethylamino) ethyl] benzene-1, 2-diol} (ISO), a synthetic catecholamine and β-adrenergic agonist has been found to cause severe stress in the myocardium resulting in infarct like necrosis of the heart muscles. Catecholamines rapidly undergo auto-oxidation and it has been suggested that the oxidative products of

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catecholamines are responsible for changes in the myocardium.[2] High concentrations of catecholamines have been reported to cause necrotic lesions in the heart resulting in myocardial infarction in experimental animals.[3]

Punica granatum (Punicaceae) fruit (commonly called Pomegranate) is considered as a heart healthy fruit juice.[4] Pomegranate is rich in antioxidant of polyphenolic class which includes tannins and anthocynins[5] and flavonoids.[6] The soluble polyphenol content in pomegranate juice is between 0.2 and1.0 %, depending on variety and include mainly tannins, ellagic tannins, anthocyanins, catechins, gallic and ellagic acids.[7] Interaction of flavonoids with various biological systems is well documented.[6] As per the epidemiological studies, cardiovascular mortality and flavonoid intake appear to be inversely proportional.[8] Despite the fact that *Punica granatum* has antioxidant properties, its cardioprotective activity against ISO induced myocardial infarction (MI) has not been studied. In view of this, the present study was designed to investigate if oral administration of *P.granatum* seed juice extract (PJ) and its butanolic fraction (B-PJ) for 21 days has any protective action against ISO-induced myocardial injury. Histopathological and biochemical changes induced by ISO have been monitored and their modulation with PJ and B-PJ were evaluated.

MATERIALS AND METHODS

Experimental animals- Male adult albino Wistar rats, weighing 150-200 g were obtained from Serum Institute Pune, India. They were housed in polypropylene cages lined with husk, renewed every 48 h under 12:12 h light dark cycle and maintained at 25^{o} ± 2^{o}C. They were fed with commercial rat pellet (AMRUT Laboratory animal feed, Pranav Agro Industries, Sangli) and given water *ad libitum*. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethical Committee.

Drugs and chemicals- Isoproterenol hydrochloride (ISO), adrenaline (Adr), noradrenaline (NA), phenylepherine (PE), serotonin (5-HT), 1,1-diphenyl, 2- picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Mumbai. All other chemicals used in the study were of analytical grade. All drug solutions were freshly prepared in saline before each experiment. The extracts were dissolved in distilled water and administered orally.

Preparation of extract- Pomegranates (1 kg) were purchased from the local market of Nashik. The seeds were separated and ground to obtain juice. The juice was then filtered and dried under reduced pressure to get pomegranate juice extract (PJ). The yield was found to be 3.2% w/w. The seeds were separated from the fruits and were refluxed with 2 M HCl for 1 h at 100^{0}C. This gave a red color extract from pomegranate which was cooled and filtered. The acidic extract was extracted with ethyl acetate to remove the flavones. This ethyl acetate fraction was heated at 80^{0}C for 3 min to remove the last traces of ethyl acetate and cooled. It was then extracted exhaustively with n- butanol. The n-butanol extract (B-PJ) (yield 1.6% w/w) was concentrated by distillation. It gave a concentrated red colored pigment which was subjected to thin layer chromatography using TLC silica gel 60 F_{254} plates (Merck) and BAW[n-butanol, acetic acid and water (4:1:5)], forestal [Concentrated HCl, acetic acid and water (3:30:10)] and formic acid [Concentrated HCl, formic acid and water (2:5:3)] as mobile phases. Iodine vapors were used for visualizing the spots on the plates and Rf values were calculated.[9] The phytochemical testing and the total flavonoid and phenolic contents of the extracts were also determined.[10-12]

Experimental design- ISO (85 mg/kg) was dissolved in normal saline and injected subcutaneously to rats at an interval of 24 h for 2 days to induce experimental MI. Animals were divided into eight groups of five each. Group I received saline, group II received ISO (85 mg/kg s.c., on 20th and 21st day), group III received PJ extract (100 mg/kg, p.o., for 3 weeks), group IV received PJ extract (300 mg/kg, p.o., for 3 weeks), group V received B- PJ extract (100 mg/kg, p.o., for 3 weeks), group VI received PJ extract (100 mg/kg, p.o., for 3 weeks) and ISO (85 mg/kg s.c., on 20th and 21st day), group VII received PJ extract (300 mg/kg, p.o., for 3 weeks) and ISO (85 mg/kg s.c., on 20th and 21st day), and group VIII received B-PJ extract (100 mg/kg, p.o., for 3 weeks) and ISO (85 mg/kg s.c., on 20th and 21stday). After 12 h following the last dose of ISO, heart rate, ECG, PRI and changes to vascular reactivity to various catecholamines were recorded using Powerlab 4SP (AD Instrument, Australia). The serum of animals of all groups was assessed for their cardiac marker enzyme levels. The heart tissue of animals of all groups was excised and subjected to superoxide dismutase (SOD) and catalase (CAT) measurements, histopathological and staining studies.

Heart rate, ECG and PRI - Needle electrodes were placed and changes in Lead II were recorded 12 h after the second dose of isoproterenol, on an electrocardiograph (Powerlab, AD Instrument, Australia). Heart rate and electrocardiograph (ECG) recordings were made in anesthetized animals for 1 min for every 5 min. The type of alterations (ST-segment elevation or depression) in normal and experimental animals was considered.[13] Pressure rate index[14] (PRI), a parameter used as an index of myocardial oxygen demand, was calculated as the product of mean arterial blood pressure (MABP) × HR/1000.

Vascular reactivity to catecholamines- After completion of

the treatment schedule, rats from each group was anesthetized with urethane (1200 mg/kg). Femoral vein was cannulated with fine polyethylene catheter for administration of the drug. Tracheostomy was performed and blood pressure (BP) was recorded from left common carotid artery using pressure transducer by direct method on Chart data system. Heparinised saline (100 IU/ ml) was filled in the transducer and in the fine polyethylene catheter cannulated to the carotid artery to prevent clotting. After 30 min of stabilization, mean change in BP to NA (1 µg/kg), Adr (1 µg/kg), Ang II (25 ng/kg) and 5-HT (1 µg/kg) were recorded.[15]

Assay of marker enzymes- At the end of vascular reactivity study, blood was collected from retro-orbital plexus from the inner canthus of the eye using capillary tubes. Serum was separated using R-24 research centrifuge (Remi Instruments Ltd, Mumbai, INDIA) at 4000 rpm for 5 min. The activities of lactate dehydrogenase-LDH[16] and creatine kinase-CK[17] were assayed in serum using commercial kits purchased from Aspen Labs, (Baddi) India and measured spectrophotometrically (Shimadzu).

Antioxidant enzymes- The heart was dissected out, immediately washed in ice-cold saline and weighed. Ten percent homogenate was prepared in 0.1 M Tris–buffer, pH 7.4. The homogenate was centrifuged at 10,000 × g for 20 min. The supernatants were used for measuring activity of enzymes -superoxide dismutase (SOD)[18] and catalase (CAT).[19]

Determination of myocardial necrosis by direct staining- Myocardium of rat was frozen immediately after removal. When the tissue was firm, the heart was sliced into 3 - 5 µm thick slice from the apex toward the atrioventricular groove and incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer saline with pH 7.4 at 37 °C for 20 min. The sections were examined under light and photographs were taken.[20]

Histopathological examination- The hearts were excised and immediately fixed in 10% buffered formalin. The ventricular mass was sectioned from the apex to the base of the heart, which was embedded in paraffin after being dehydrated in alcohol and subsequently cleared with xylene. Five-micrometer thick serial histological sections were obtained from the paraffin blocks and stained with hematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.[21]

Statistical analysis- Data are presented as mean ± SEM. The data were analyzed by one-way ANOVA followed by Dunnett's test.

RESULTS

Phytochemical investigations of PJ and B-PJ- The total flavonoid content of PJ-extract and its n-B-PJ extract was found to be 455.7 ± 6.33 µg and 222.7 ± 2.88 µg rutin equivalent/ mg of extract, respectively. The total phenolic content of PJ-extract and its n-B-PJ extract was found to be 94.15 ± 9.92 µg and 56.56 ± 3.58 µg gallic acid equivalent/mg of extract, respectively. The phytoconstituents present in the PJ extract were flavonoids and tannins whereas B-PJ extract showed only flavonoid positive. The Rf values for B-PJ using BAW, forestal and formic acid were 0.86, 0.71 and 0.40, respectively, which indicates the presence of anthocynidins.

Effect of PJ extract on heart rate, pressure rate index and ST elevation in ECG- The heart rate, PRI and ECG in vehicle-treated animals was recorded as 282.5 ± 8.54 beats/ min, 25.65 ± 1.97 mm Hg/min and 0.029 ± 0.001 mV, respectively. ISO-treated rats showed a significant ($P < 0.05$) elevation in heart rate, PRI and ST segments. Normal rats treated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) did not show any significant change in parameters. Pretreatment with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) in ISO-treated animals restored the heart rate to near normal and showed a moderate elevation in PRI and ST segments [Table 1].

Effect of PJ extract on vascular reactivity- In vehicle-treated animals, the mean change in blood pressure induced by Adr (1 µg/kg), NA (1 µg/kg), Ang II (25 ng/kg) and 5-HT (1 µg/kg) was 13.75 ± 2.17, 23.8 ± 1.74, 16.4 ± 1.12, and 19.5 ± 2.45 mm Hg, respectively. Isoproterenol treatment significantly

Table 1: Effect of PJ and B-PJ on cardiac parameters

Treatment group (mg/kg)	Heart rate (beats/min)	MABP (mmHg)	Pressure rate index (mmHg/min)	ST elevation (mV)
Control	282.5 ± 8.54	90.70 ± 4.94	25.65 ± 1.97	0.029 ± 0.001
ISO (85)	500.4 ± 13.52*	115.75 ± 1.85*	57.92 ± 1.48*	0.083 ± 0.009*
PJ (100)	343.0 ± 17.48	100.61 ± 2.40	34.51 ± 2.15	0.036 ± 0.007
PJ (300)	302.2 ± 16.93	91.09 ± 5.58	27.53 ± 2.32	0.027 ± 0.0017
B-PJ (100)	305.7 ± 8.09	100.70 ± 3.42	31.12 ± 1.65	0.018 ± 0.0035
PJ (100)+ ISO (85)	430 ± 30.07#	104.55 ± 8.74#	44.96 ± 3.74#	0.064 ± 0.0045
PJ (300)+ ISO (85)	368.0 ± 19.53#	93.83 ± 3.60#	34.53 ± 1.21#	0.051 ± 0.005#
B-PJ (100)+ ISO (85)	397.5 ± 17.5#	96.55 ± 2.75#	38.38 ± 2.28#	0.32 ± 0.0025#

N=5, All values are expressed as mean ±SEM. $P^* < 0.05$ against Control group, $P\# < 0.05$ against isoproterenol group, One-way ANOVA followed by Dunnett's test

increased ($P < 0.05$) the mean change in BP as compared to their respective controls. Treatment with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) alone did not produce any significant change when compared to vehicle-treated animals. Pretreatment with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) showed a significant decrease in mean change in BP as compared to ISO-treated group [Figure 1].

Effect of PJ extract on cardiac marker enzymes-The serum LDH and CK concentrations in vehicle-treated rats were 78.75 ± 2.6 and 4.27 ± 0.68 IU/L. Rats treated with ISO showed a significant ($P < 0.05$) increase in the activities of these enzymes in serum. Rats treated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) alone did not alter the cardiac marker enzyme levels in serum as compared to vehicle-treated rats. Pretreatment with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) in ISO-treated rats significantly ($P < 0.05$) reduced the activities of enzymes as compared to ISO-treated rats [Figure 2].

Effect of PJ extract on antioxidant enzymes-Table 2 shows the activities of antioxidant enzymes- SOD and CAT. The antioxidant enzyme activities were decreased significantly ($P < 0.05$) in ISO-treated rats when compared to those of control rats. The activities of antioxidant enzymes were maintained at near normal in animals pretreated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) as compared to ISO-treated rats. Normal animals treated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) alone did not significantly alter the antioxidant enzyme levels.

Effect of PJ extract on TTC staining and histopathology-The heart tissues of control and PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) treated group were stained brick red (dark region) with TTC, an indicator of mitochondrial respiration. The unstained region is an indicator of total necrosis as seen in ISO-treated group. The sections of heart tissues pretreated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) in ISO group showed a lesser degree of unstained region compared to ISO-treated group [Figure 3]. In histopathological examination, normal architecture was observed in control animals whereas animals treated with ISO showed thrombus formation, contraction band necrosis and inflammation. Animals pretreated with PJ (100 mg/kg, p.o. and 300 mg/kg, p.o.) and B-PJ (100 mg/kg., p.o.) revealed much less intensity of the above changes [Figure 4].

Table 2: Effect of PJ and B-PJ on antioxidant enzymes

Treatment group (mg/kg)	SOD (U/mg of wet tissue)	CAT (×10-3U/mg of wet tissue)
Control	64.1 ± 2.99	12.31 ± 0.80
ISO (85)	26.38 ± 1.98*	3.95 ± 0.16*
PJ (100)	52.62 ± 1.99	10.2 ± 0.51
PJ (300)	58.82 ± 4.46	11.77 ± 0.23
B-PJ (100)	56.77 ± 2.90	17.19 ± 0.31*
PJ (100)+ ISO (85)	32.51 ± 1.23	4.4 ± 0.4
PJ (300)+ ISO (85)	44.44 ± 5.39#	8.0 ± 0.5#
B-PJ (100)+ ISO (85)	44.5 ± 3.53#	7.99 ± 1.27#

N=5, All values are expressed as mean ±SEM. p* < 0.05 against Control group, *P*# < 0.05 against isoproterenol group, One-way ANOVA followed by Dunnett's test.

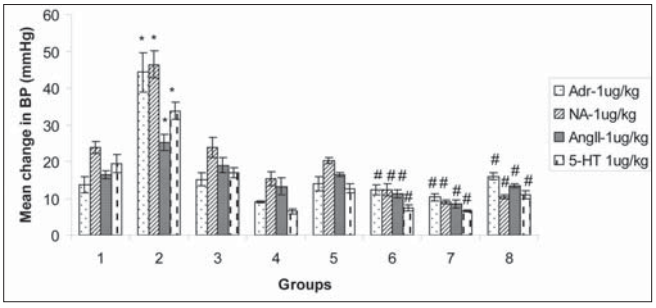

Figure 1: Effect of PJ (100 mg/kg and 300 mg/kg, p.o., for 3 weeks) and B-PJ (100 mg/kg, p.o., for 3 weeks) on vascular reactivity to Adr (1µg/kg), NA(1µg/kg), AngII (25ng/kg), and 5-HT (1µg/kg) in ISO (85mg/kg, s.c., at interval of 24 h for 2days) induced MI N=5, All values are expressed as mean ±SEM. *P** < 0.05 against Control group, p# < 0.05 against isoproterenol group, One-way ANOVA followed by Dunnett's test

DISCUSSION

In the present study, administration of PJ and B-PJ extracts showed a protective effect against ISO-induced alterations in various cardiac, biochemical and histopathological parameters. Pre-treatment with PJ extract (100 and 300 mg/kg) and B-PJ (100 mg/kg) for 21 days caused restoration of heart rate, PRI and ECG values to normal, a significant reduction in vascular reactivity to various agonists, a significant increase in the levels of SOD and CAT and a significant decrease in the levels of LDH and CK as evident by the gross morphologic and histopathologic changes.

Isoproterenol administration in large dose induces morphological and functional changes in the heart leading to

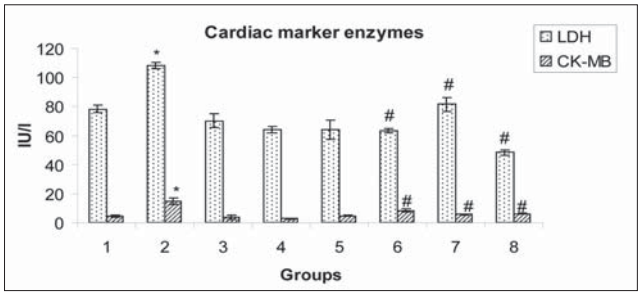

Figure 2: Effect of PJ (100 mg/kg and 300 mg/kg, p.o., for 3 weeks) and B-PJ (100 mg/kg, p.o., for 3 weeks) on LDH & CK-MB in ISO induced MI N=5, All values are expressed as mean ±SEM. *P** < 0.05 against Control group, *P*# < 0.05 against isoproterenol group, One-way ANOVA followed by Dunnett's test

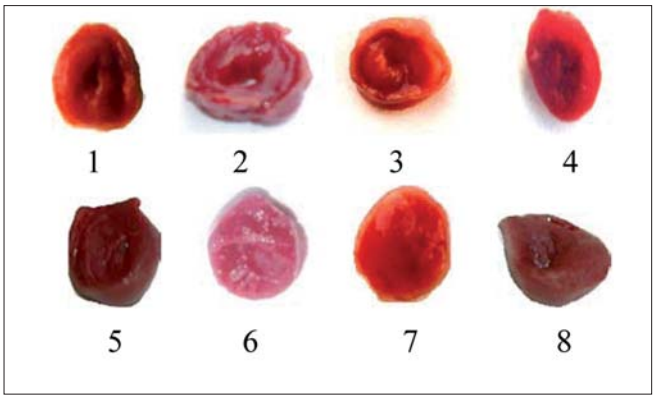

Figure 3: Photographs of transverse sections of heart from control and experimental groups stained by triphenyltetrazolium chloride 1: group1; 2: group 2; 3: group 3; 4: group 4; 5: group 5; 6: group 6; 7: group 7; 8: group 8.

myocardial necrosis.[22] The toxic effects of catecholamines could be accounted due to the oxidation of hydroxyl groups in catecholamines leading to the conversion into quinones and the subsequent formation of adrenochromes which cause cell necrosis and contractile failure in the rat's heart. Highly toxic oxygen-derived free radicals are produced during this phase which is detrimental to extra- and intracellular enzymes and proteins.[23] ECG-abnormalities is an important tool for the accurate diagnosis of MI.[24] It is reported that ST elevation correlates well with the leak of CK from the myocardium and the degree of damage observed histologically.[25] Pressure rate index was calculated as an index of oxygen demand in all groups. Our results are in congruence with the previous study as reported by Kela *et al* and Satish. *et al*.[26,27] Vascular reactivity to NA, Adr, AngII and 5-HT was measured by invasive blood pressure technique in all groups of rats, as described earlier by Balaraman *et al*. Endogenous enzymes such as catalase and superoxide dismustase are the first line cellular defense free radical scavenging enzymes against oxidative injury.[28] Increased activity of SOD and CAT indicates increased removal of superoxide radicals thereby reducing myocardial damage caused by free radicals.

It has been reported that, TTC forms a red formazan precipitate with LDH of the viable myocardial tissue in the presence of mitochondrial dehydrogenase enzyme system, whereas areas of necrosis lack mitochondrial dehydrogenase activity and do not stain. Consequently, areas not stained with TTC correspond to areas of total necrosis.[29]

On histopathological examination, ISO-treated group, demonstrated thrombus formation, contraction band necrosis and inflammation. Pretreament with PJ extract and B-PJ reversed these changes. It is concluded that PJ extract and its butanolic fraction has a potential to inhibit the cardiotoxic effects induced by ISO and possesses a significant therapeutic value in the prophylactic treatment of MI.

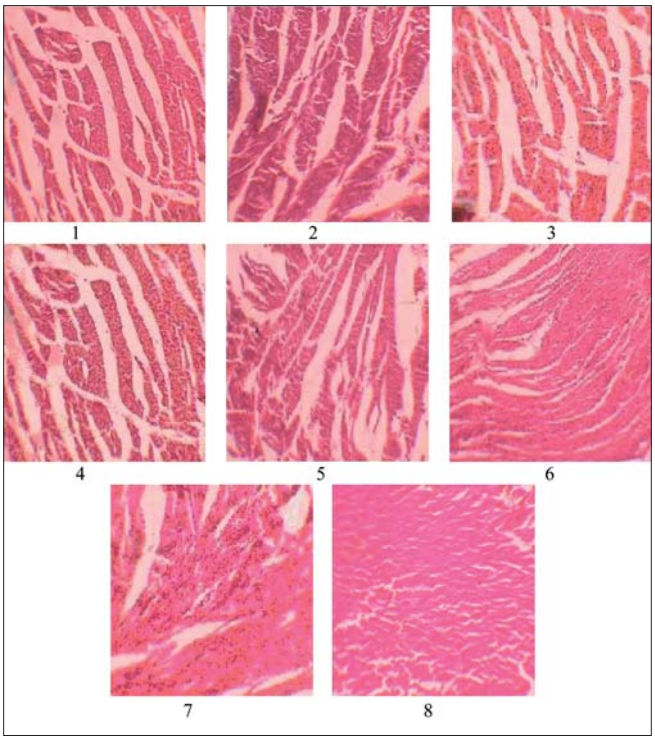

Figure 4: Photomicrographs of histopathological examination (10×) of the heart from control and experimental groups Section of the heart from 1 (group-1) shows normal architecture. Section of the heart from 2 (group 2) reveals thrombus formation, contraction band necrosis and inflammation. Sections of heart from 3 (group 3); 4 (group 4) and 5 (group 5) shows normal architecture, whereas. Sections of heart from 6 (group 6); 7 (group 7) and 8 (group 8) shows less intensity of congestion, thrombus formation and necrosis.

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