Effect of 15 days hindlimb unloading on baroreflex sensitivity, Hear rate variability and Carotid artery structure in rats

Abstract:

Introduction: In space, cephalad shift of body fluid equalizes the blood pressure throughout the body leading to various cardiovascular disturbances. Effects of microgravity have been perfectly simulated in the rat hindlimb unloading (HU) model. This study was planned to assess the structure-function relationship of the carotid artery before and after HU of 14 days

Material and methods: This study was done in Department of Physiology, AIIMS, New Delhi. 18 male Wistar rats weighing 250-300 gm were randomly divided into control and hindlimb unloaded groups and housed in separate cages for 14 days. Body weight, food and water intake was measured every day. The pelvic suspension method was used to simulate microgravity. Blood pressure was recorded directly from the carotid artery.ECG was recorded in lead II configuration. A graph was plotted of MAP v RRI and the slope was taken. Linear regression of mean arterial pressure and RR interval used to derive the Baroreflex sensitivity.Vessel wall thickness, intimal thickness, smooth muscle cell layer thickness & luminal diameter were observed after H&E staining. Ultrathin sectioning and counter-staining was done for electron microscopy and changes in endothelial thickness, elastic lamina, and deeper artery structures were observed. Statistical analysis was done using Graphpad Prism (version 9.0). Unpaired t-test was applied.

Results:

We found significant differences were observed Food intake($p < 0.001$) Body weight($p < 0.001$), Heart rate ($p=0.031$), BRS ($p= 0.026$), HRV:R-R interval ($p=0.0246$), SDSD ($p=0.0001$),RMSD ($p= 0.0001$),VLF($p=0.001$),HF ($p=0.0054$), LF/HF Ratio ($p= 0.0001$).we did not found significant changes inWater intake, MAP, SBP, DBP, LFT, RFT, Vessel wall thickness(µm), Luminal Diameter (µm), Intimal thickness(µm), Smooth muscle thickness(µm), in H&E staining and in electron microscopy.

Conclusion:

Chronic microgravity simulation using pelvic suspension showed a decrease in BRS, however 15 days are not insufficient to produce any structural changes in the carotid artery. BRS changes are accompanied by change vascular vasoconstriction and vasodilation properties which are due to alteration in the sympathetic and parasympathetic outflow.

Key Words: Microgravity, Hind limb suspension, Pelvic suspension, Baroreflex sensitivity, Heart rate variability.

Introduction:

On earth, the earth's gravity creates a blood pressure gradient in the upright position in the human body as a result resting blood pressure in the head is 70 mmHg & in the foot 200 mmHg respectively. When astronaut goes in the space, this blood pressure gradient vanishes and creates uniform blood pressure. Effects of microgravity exposure on cardiovascular system include fluid redistribution, fluid loss, decrease stroke volume, decreased aerobic capacity, cardiac atrophy and orthostatic hypotension upon return to earth. (1,2) Effects of microgravity on the cardiovascular system have been studied well in the astronauts but due to limitations of human studies, many laboratories like NASA have used the rat hindlimb unloading (HU) model to simulate effects of microgravity. (3-4)

HU models have perfectly simulated effects of microgravity such as cephalic fluid shift, weight loss, disuse atrophy of muscles, bone loss, tachycardia, and BP changes. By HU model, effects of Microgravity have been studied in both acute and chronic conditions by unloading the rats at different angles (20 to 45˚) and for the duration (one day to months). Acute microgravity simulation using tail suspension showed a decrease in BRS which returned to baseline after 48 hours of recovery. (5) The chronic microgravity simulation using tail suspension for 2 weeks showed abnormal baroreflex response during tail suspension (6) Raffai et al. 2008 showed simulated microgravity in rats by head down tit (HDT) for 7 days resulted in BP elevations and baroreflex sensitivity reduction in the HU group (7). On the contrary, Brizzee et al. 1990 (HU for 7 days) Fagette et al. 1995 (HU for 13 days) did not find any change in BRS. (7, 8)

Exact mechanism of change in BRS is not known, structural changes in major arteries due to chronic cephalic fluid shift after HU might be the mechanism for the change in baroreceptor sensitivity, heart rate, and blood pressure, which was studied by Chew et al. (1997). They did HU for 10-14 weeks of HU and they found an increase in arterial thickness in the femoral, carotid, axillary and iliac arteries. However, Gao et al. (2008) did not find any changes in vessel after tail suspended head-down tilt for 28 days. (9, 10)

Above mentioned literature suggests that the effect of microgravity simulation by HU has yielded varying results on baroreflex sensitivity & carotid artery structure, and carotid artery structural changes have not been studied in conjunction with the changes in baroreflex sensitivity after HU. So, the hypothesis of the study was that chronic HU might lead to structural changes in the carotid artery which manifest as a change in BRS & HRV. Thereafter

this study was planned to assess the structure-function relationship of the carotid artery before and after HU of 14 days.

Materials and methods:

This study was done in the Department of Physiology, AIIMS, New Delhi. We received ethical clearance from the Institutional Animal Ethics Committee, AIIMS (File No. 153/IAEC-1/2019). The experiments were performed in accordance to the National Institute of Health (NIH) Guidelines for the Care and Use of the Laboratory Animals (NIH Publication no. 85723, revised 1996). 18 adult male Wistar rats (250-280 gm) were procured from the Central animal facility, AIIMS, New Delhi. Each rat was housed individually in polypropylene cages and provided with laboratory food pellets and clean drinking water ad libitum . All the animals were housed and maintained at an ambient room temperature of 24±2˚C, relative humidity of 50-55%, and the light-dark cycle of 12:12 hours. After the initial acclimatization period rats were randomly divided into 2 groups; control and hind limb suspension. Hind limb suspension rats were suspended in the cage using a plastic wire which was passed through the brace. Suspension cage was fabricated similar to the cage used by Morey-Holton with certain modifications. (11)

Hip Suspension of rats

The pelvic suspension method was used to simulate microgravity. A hip brace was fabricated by modifying the brace described by Chowdhary et al 2013. (12) The metallic brace covered with leucoplast were curved in the shape of the pelvic brace. Once the pelvic brace was ready, the rat was anesthetized using ketamine & xylazine and it was fitted into the brace. The brace was tightened to ensure that the rat was not able to escape. Rats were allowed to acclimatize to the brace for 7 days, and then rats were suspended at an angle of 30 degrees bypassing plastic insulated wires through the brace. (Fig.1)

Recording of Body weight, food intake, and water intake:

The body weight of rats was taken every day by a weighing machine (range: 0-500g, sensitivity: 10 gm). The rats were given standard rat chow and Food intake was measured using weighing methods. To calculate water intake, 100 ml of water was given to the rat every day at 10 am and the next day at 10 am remaining water was marked.

Baroreflex sensitivity recording

The rats were anesthetized using urethane (1.5gm/Kg) by intraperitoneally route and depth of anesthesia was checked by withdrawal reflex. Then the rat was placed ventrally on surgical

table and after shaving the fur from the anterior area of the neck, the surgical area was cleaned using betadine. To record heart rate, ECG leads were attached in lead II configuration. 0.5 cm midline skin incision was made by surgical scissor, followed by blunt dissection of glands, subcutaneous tissue, and omohyoid muscle on the side of the trachea by two glass seekers. The carotid artery was identified as a big palpating artery with a vagus nerve on the side of the trachea. The vagus nerve was separated from the artery carefully, and then the carotid artery was cannulated by pre-heparinized 24 gauge venflon. Immediately after cannulation, 0.5 ml of heparinized saline was slowly flushed in the heart to prevent clotting. Then the fluid-filled pressure transducer (Power lab, ADInstruments) was connected to the cannula. (Fig.2) Baseline blood pressure and heart rate were recorded. After 30 minutes of baseline recording, blood pressure and heart rate response to four doses each of phenylephrine (5,10, 15 & 20 µgm/kg) and sodium nitroprusside (6,12, 24 & 48 µgm/kg) in a randomized manner were recorded. Following each dose, enough time was allowed to the blood pressure and heart rate to return to baseline. (13)

Histology:

After blood pressure recording, rats were immediately scarified and perfused with normal saline. Then the carotid artery was dissected and placed in a paraformaldehyde solution for fixation. Fixed Samples were stored in a 4°C for 24 hours. Stored tissues were then sequentially transferred to sucrose solutions in the next 2 days, followed by 8 micrometer thick sectioning by cryostat microtome (Leica, Germany) and staining by hematoxylin and eosin. The imaging was done using the NIS Element basic research software. Vessel wall thickness, intimal thickness, smooth muscle cell layer thickness & luminal diameter were assessed by the Fiji ImageJ software.

Electron microscopy:

The carotid artery was dissected and placed in the mixture of 2% Paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer saline at 4°C for 24 h. Secondary fixation of the artery was done in the mixture 1% osmium tetroxide and 1.5% potassium ferrocyanide at 0°C for one hour. After that tissue was transferred for an additional one hour in 1% OsO_4 in 0.1 M sodium cacodylate. Then the tissue was dehydrated with different concentrations of ethanol. Tissue infiltration was done by propylene oxide: Durcupan (1:1), then tissue embedding by Durcupan for 48h. To select areas for ultrathin sectioning (70-80nm), a semi-thick tissue section (500nm) was first cut and sections were microscopically evaluated. Tissue sections with good visibility of all the vessel characteristics were then subjected to ultrathin sectioning. Ultrathin sections (100nm) were cut by Ultracut S microtome (Leica) and counter-staining was done by lead

citrate, then sections were observed under a Tecnai G2 20 electron microscope (Fei Company, The Netherlands). Images were digitally acquired at 750-2250 X magnification by a charge-coupled device (CCD) camera.

Statistical analysis

Statistical analysis was done using Graphpad prism (version 9.0). Data was first tested for distribution using the Shapiro-Wilk normality test. According to distribution Unpaired t test or Mann Whitney were used. To calculate BRS, different doses of phenylephrine (5,10, 15 & 20 µgm/kg) and sodium nitroprusside (6,12, 24 & 48 µgm/kg) were given through carotid artery and HR rate and the MAP was recorded. The MAP and R-R Interval are plotted and the slope of the graph is calculated and taken as the Baroreflex sensitivity. To derive baroreflex sensitivity linear regression was done between RR interval and mean arterial pressure change.

Results:

Changes in the bodyweight of both the groups of rats were compared at end of 1st day, 5th day, 10th day, and 14th day. There was a significant weight difference in between pelvic suspension group and the control group at 10th day (p value <0.001) and 14th day (p value <0.001). [(Baseline, C: 264.16±4.55 vs HU: 265.83±5.23)], [(Day 1, C: 263.33±4.95 vs HU: 261.67±4.77)], [Day 5, (C: 268.33±4.77 vs HU: 251.67±4.01)], [Day 10, C: 275±4.28: vs HU: 241.67±4.01) and [Day 14, C: 278.33±5.43: vs HU: 241.67±3.07). (Fig. 3)

Difference in the food intake in both the groups of rats was compared at the end of 1st, 5th, 10th and 14th day (Fig.2). There was significant less food intake (p value <0.001) in pelvic suspension group as compared to control group at 5th, 10th and 14th day.). [(Baseline, C: 35±3.16vs HU: 30±4.49)], [(Day 1, C: 34±2.98 vs HU: 22.17±2.66)], [Day 5, (C: 36.17±8.61 vs HU: 19.17±3.76)], [10th, C: 37.33±8.57vs HU: 30±3.47] and [14th, C: 43.33±4.69 vs HU: 28.5±3.03]. Figure 2 shows a difference in the water intake in both the groups of rats at the end of 5th, 10th and 14th day. There was no significant difference in water intake in pelvic suspension group as compared to control group (p value <0.001).). [(Day 0, C: 43.33±5.05 vs HU: 42.5±8.22)], [(Day 1, C 40±4.75: vs HU: 35.83±3.59)], [Day 5, (C: 43.33±5.05 vs HU: 40±3.23)] and [10th day {46.67±8.76 vs HU: 41.5±3.62) and [10th day, (53.33±4.10vs HU: 50.83±2.44). (Fig. 3)

Difference in the MAP and BP of both the groups of rats were compared at the end of 14th day respectively (Fig. 2). There was no significant difference in between pelvic suspension group and control group at 14th day. MAP at 14th day [C: 90.48±8.37mmHg vs Ex: 91.77±8.97mmHg (p=0.801)], SBP 14th day [C: 105.6±10.02 mmHg vs HU: 108.4 ±7.5 mmHg (p=0.801)]. Fig. 3

shows HR of both the groups of rats at the end of 14th day. There was a significant difference in HR between pelvic suspension group and control group on 14th day. [C: 223.2±22.3/min vs HU: 279.9±0.75/min (p=0.031)]. Fig. 3H shows the RR interval of both the groups of rats at the end of the 14th day. There was a significant difference in HR between pelvic suspension group and control group on 14th day. [C: 223.2±22.3/min vs HU: 279.9±0.75/min (p=0.031)]. Blood pressure responses to different doses of phenylephrine (5, 10, 15 & 20 µgm/kg) and sodium nitroprusside (6, 12, 24 & 48 µgm/kg) were recorded. There was significant lower blood pressure elevation to different doses of epinephrine in the pelvic suspension group as compared to control whereas significant lower blood pressure falls to different doses of nitroprusside in the pelvic suspension group. (Fig. 3) We also found no significant correlation between BRS with Vessel wall thickness, luminal diameter, Intimal thickness Smooth muscle layer thickness.

To examine the structural changes in the carotid artery, H & E staining was done and the transverse section of the vessel was examined in different magnification. (Fig.5) Different comparative parameters such as Vessel wall thickness, luminal diameter, Intimal thickness & Smooth muscle layer thickness of both the groups of rats were assessed and measured. Fig. 3 show the Vessel wall thickness, luminal diameter, Intimal thickness & Smooth muscle layer thickness of both the groups of rats at the end of 14th day respectively. There was a trend of increase in the vessel wall thickness, Intimal thickness & Smooth muscle layer thickness in the pelvic suspension group but it was not significant; similarly non-significant reduction in luminal diameter in pelvic suspension group was observed. Vessel wall thickness [C: 125±23.34 µm vs HU: 146.5±12.05 µm (p=0.072)], luminal diameter [C: 2046±374.1µm vs HU: 1913±235.2 µm (p=0.475)], intimal thickness [C: 39.22±15.98 µm vs HU: 44.84±8.991 µm (p=0.47)], Smooth muscle layer thickness [C: 96.97±24.71µm vs HU: 114.5±17.88µm (p=0.189)] (Fig 3 & 5) we did not found any significant change in endothelial thickness, no of smooth muscle and elastic lamina thickness in electron micrograph of carotid artery of rats. (Fig. 5, 6)

To examine the vasodilator and vasoconstriction properties of the carotid artery, cannulation was done and different doses of epinephrine and nitroprusside were used. Fig. 3 shows the change in the mean arterial blood pressure for the different doses of epinephrine and nitroprusside respectively. [Epinephrine 6 µgm/kg (C: 26.82±4.74 ±4.74 vs HU:18.21±6.21), 12 µgm/kg (C:50.26±7.01 vs HU:37.06±7.38), 24 µgm/kg (C:63.97±5.9 vs Ex:52±4.3), 24 µgm/kg (C:71.21±7.4 vs HU: 58.51±4.7) (p=0.0001)], Nitroprusside 6 µgm/kg (C: -30.33±7.22 vs HU:-22 ±5.5), 12 µgm/kg (C:50.26±5.25 vs HU:-36±6.16), 24 µgm/kg (-65.7±7 vs -51.6±6.66), 24 µgm/kg (E:-72±64 vs HU: -61.66±6.4) (p=0.0001)]. We have compared the dose response curve for different dose of epinephrine and nitroprusside which showed after HU, IC50 were shifted

significantly on right side for epinephrine and left side for nitroprusside. IC50 for Epinephrine (C: 2.57 vs HU: 3.17), nitroprusside (C: 7.6 vs HU: 11.6). (Fig 4)

Changes in the BRS were not accompanied by changes vascular structure, so it might be due to alteration in sympathetic and parasympathetic outflow. So we planned to compare autonomic tone of different group of rats by heart rate variability. To examine the effect chronic HU on sympathetic and parasympathetic system, we have done the HRV of both the group of rats. After 30 min of lead 2 ECG recording, we found significant change in R-R interval, SDSD, RMSD, VLF and HF power and LF/HF ratio between control and HU group after 14 days of HU. (Fig 7)

Discussion:

We have simulated chronic microgravity in rat model by pelvic suspension method. We found significant changes in the body weight, food intake, water intake, HR, BRS, vasodilator & vasoconstrictor response.

The body weights of the HU rats were significantly low compared to the control rats. We hypothesize that the reduction in the body weight is a direct consequence of the decrease in food intake But recent study by Jin et 2018 showed that simulated weightlessness could modify the intestinal permeability (15) Whereas Shimizu et al. 1999 reported that the changes in body weight in rats might be due to the space environment (16). As the suspension period came to an end, food and water intake showed a trend towards increase. This could be attributed to habituation of rats after a few days of suspension with the pelvis brace. Our experiments demonstrated significant changes in water intake in pelvic suspended rats after 15 days of suspension. Many studies have compared the water intake after HU but finding shows variable results such as Steffen et al 1990 showed a significant decrease in water intake where as Martel et 1996 found no significant reduction in water intake after HU.(17,18) one of the important factors that could affect water intake was hypokinesia. We hypothesised that hyporkinesia might be responsible for the decrease in energy expenditure which leads to decrease in water and food intake.

We did not found any significant change in hemodynamic parameters such as SBP, DBP and MAP in HU group as compare to the control group. In human/rats studies blood pressure increases might be due to stress after exposure to microgravity. (19) Some of these differences between humans and rodents may reflect the differences between circulatory adjustments in bipeds and quadrupeds or the differences in large and small mammals (20). The fact that fluid shifts from lower body to upper play an important role in the adaptation to microgravity, the

small body size of rodents may be a limiting factor for these adaptations. In our study we found that the heart rate of the pelvic suspended group showed a significant increase. Human studies during Spacelab missions demonstrated that heart rate was elevated due to exposure to microgravity (21, 22). Prolonged exposure to microgravity has been shown to increase HR (23). Postflight quiet standing heart rate is markedly elevated (19). In rats, heart rate response post suspension has been found variable. (24)

We found a significant decrease in the BRS in the pelvic suspension group. This decreased range of baroreflex buffering suggests that there is either a diminished capacity to increase heart rate by alteration in vagal tone or sympathetic responsiveness. After a 4- to 5-day space shuttle mission, investigators found reductions in carotid baroreceptor-reflex responsiveness (25). In astronauts who could not finish the Postflight stand test, heart rate failed to continue to increase with progressive hypotension, suggesting a reduced range of baroreflex buffering. After longer missions (8–14 days), baroreflex slope, total range, and operational point were reduced post flight, and the degree of this reduction correlated with the degree of orthostatic intolerance (26).We found an increasing trend in the vessel wall thickness. Intimal thickness, smooth muscle layer thickness and the luminal diameter but these changes were non-significant changes. Similar findings were reported in other studies e.g. Gao et al 2009 that there was an increase in the media thickness of the common carotid artery and basilar artery after 28 days of TS. (10) In some of the previous studies, vascular adaptation to microgravity has been suggested to be a generalized or universal response (27) whereas others suggest regional phenomena. (28)

Pressure may be the primary stimulus initiating regional adaptation in arteries. In With respect to vascular adaptation to microgravity, redistribution of transmural pressure across the vasculature seems to be the primary stimulus and the redistribution of flow and volume is a secondary consequence. This pressure redistribution is maintained as long as head-down tilt or microgravity exposure is continued, even though the body fluid redistribution has attained a new equilibrium. Although many local mechanical and growth factors, such as shear stress, growth factors, cytokines, etc. (29). We did not found any significant correlation between BRS and vascular structural parameter, which suggests that change in BRS happens before the change in carotid artery structure due to pelvic suspension.

Based on these data, we propose that due to the chronic cephalic shift of body fluid in the blood vessels located above the heart lead to resetting of the Baroreceptor operation point and range which lead to less Baroreceptor response in the rats after 14 days of pelvic suspension. If the exposure to microgravity continues for a longer duration, the above phenomena becomes more stable and the muscle cells in the carotid arteries that are being continuously subjected to

a higher pressure might show hypertrophy to compensate for high pressure which we could not get might be due to short duration of intervention. We also found impairment in VLF power in HRV which suggests impairment in rennin angiotensin Aldosterone system that should evaluated with larger duration of HU.

Conclusion:

We found that 15 days of microgravity simulation using pelvic suspension lead to decrease in Baroreceptor sensitivity however it is not sufficient to produce any structural change in the carotid artery. Changes in the BRS are accompanied by and vascular vasoconstriction and vasodilation properties which is due to alteration in sympathetic and parasympathetic outflow.

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Fig 1 (A) Suspended rat (B) Pelvic brace for pelvis suspension (C) Cage for housing of test rats.

Fig 2: Steps of procedure of invasive blood pressure recording from carotid artery in rat

Fig 3: Graphical representation of comparison of between control and HU rats

Fig 4: Dose response curve for different dose of epinephrine and nitroprusside

Fig 5: Carotid artery histology in different magnification (H & E staining)

Fig 6: Electron microscopy of carotid artery ultra structure in different magnifications. EN (endothelial layer), EL (elastic lamina), SM (smooth muscle)

Fig 7: Comparison of Heart rate variability between control and HU rats

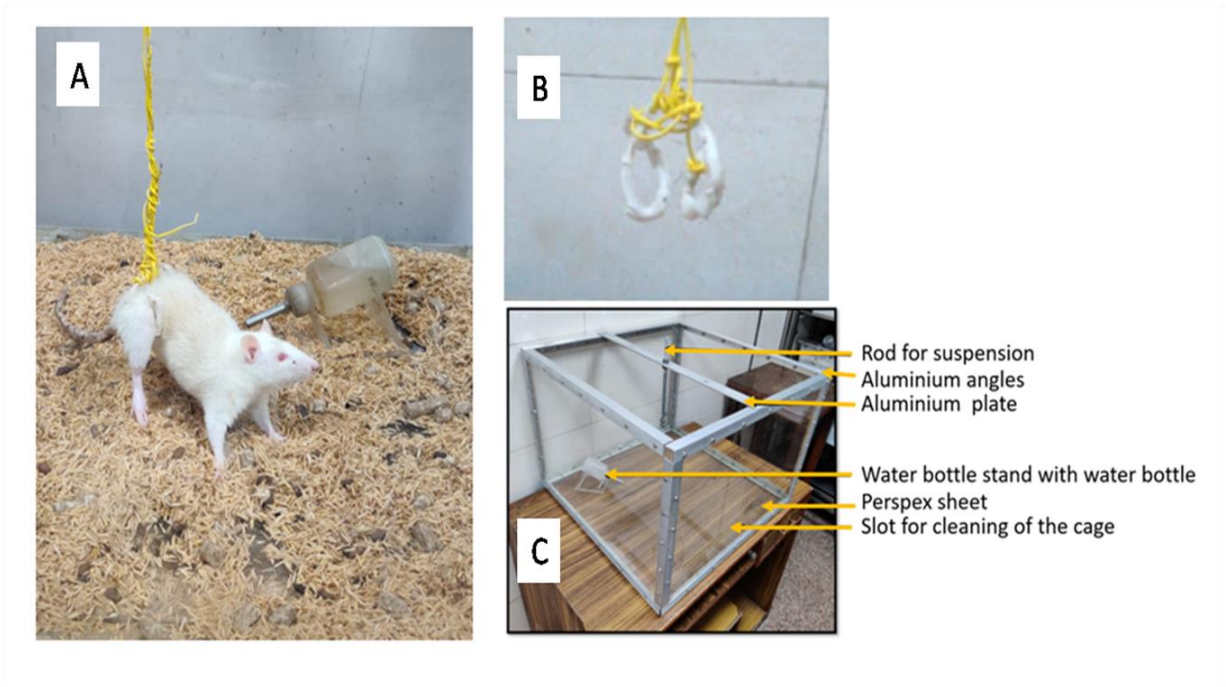

Figure 1: (A) Suspended rat (B) Pelvic brace for pelvis suspension
(C) Cage for housing of test rats.

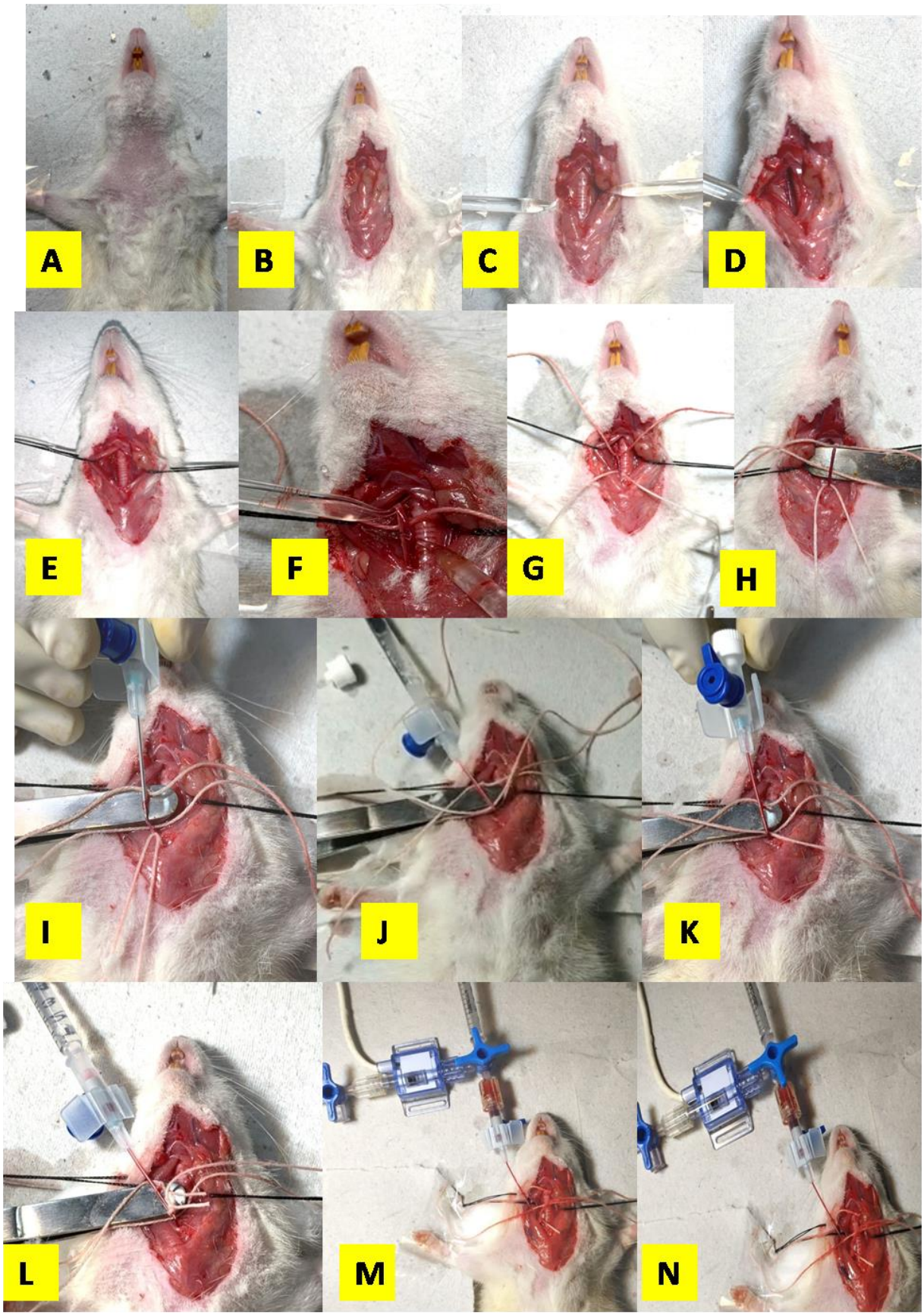

Fig.2: Surgical procedure of recording of BRS.

A: Shaving of chest of the rat. B: Thymus and other glands

Fig.2: Surgical procedure of recording of BRS.
A: Shaving of chest of the rat. B: Thymus and other glands visualization after removal of skin in the chest. C: Visualization of trachea. D: Visualization of vagus nerve and carotid artery in the side of trachea. E: side muscles are sutured to exposed the surgical area. F: Vagus nerve were dissected out from carotid sheath. G: 2 Threads were passed behind the carotid artery. H: A support is given from behind to the carotid artery for cannulaltion. I: A nick is made by 16 gauze venflon. J: Trocar of venflon is removed and heparinised saline was injected in artery to prevent clotting. K: Venflon is closed by cap and cannulaltion is secured by a knot. L: heparinised saline was injected in artery to prevent clotting. M: fluid filled pressure transducer was attached to cannula and baseline BP was recorded. N: different dose of phenylepinephine and nitroprusside was injected and BRS were recorded for the same.

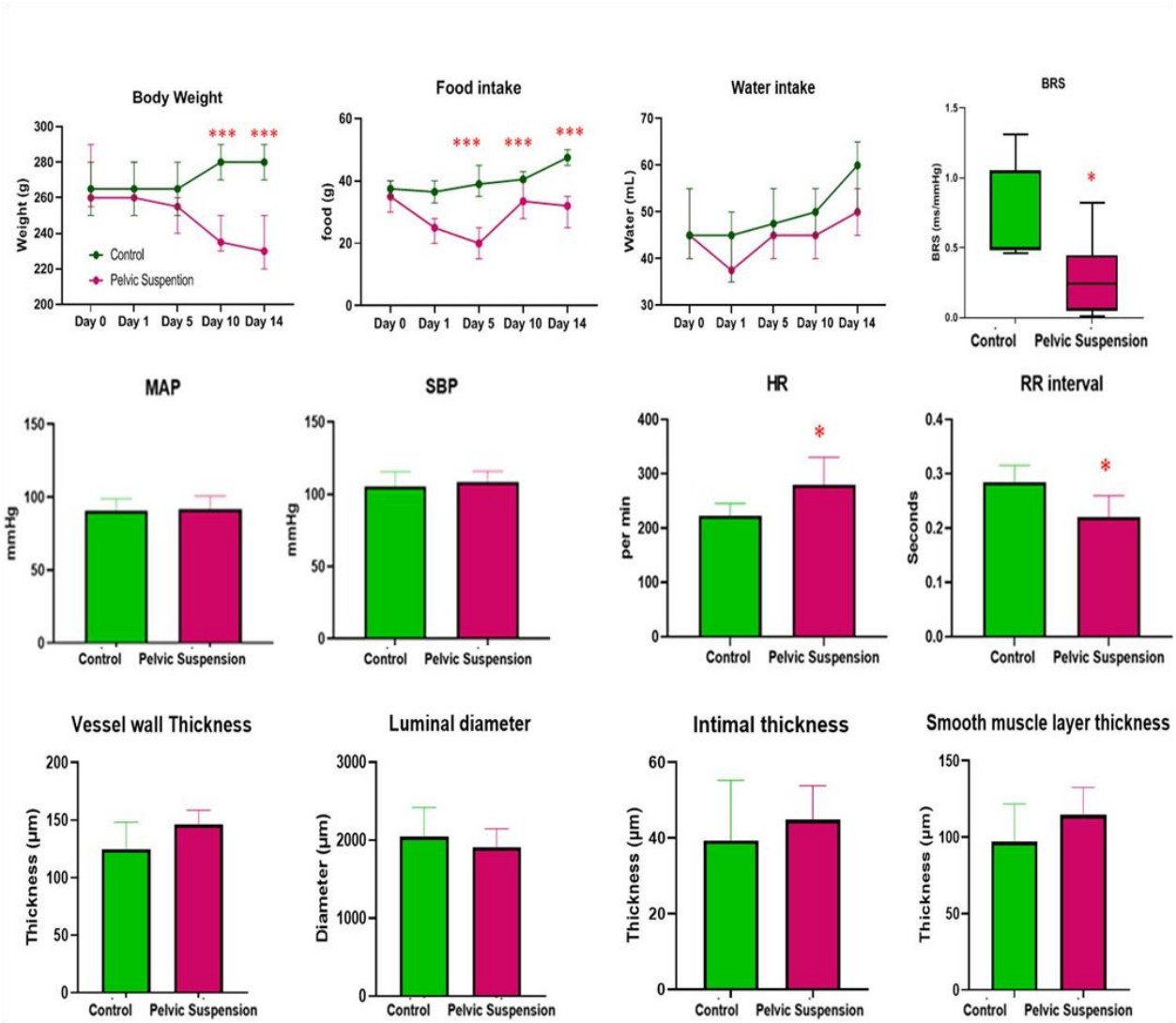

Fig 3: Graphical representation of comparison of cardiovascular and carotid artery structures between control and HU rats

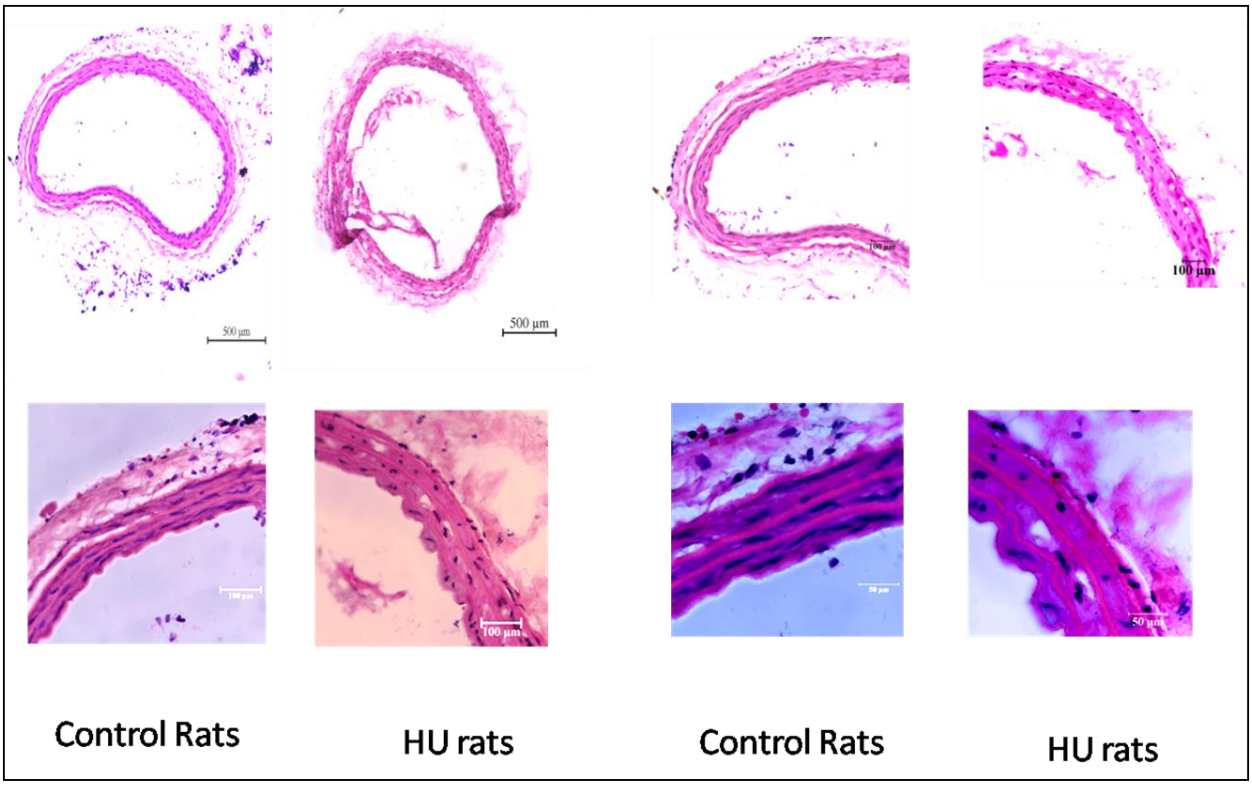

Figure 4: Carotid artery histology in different magnification (4X, 10X, 20X & 40X)

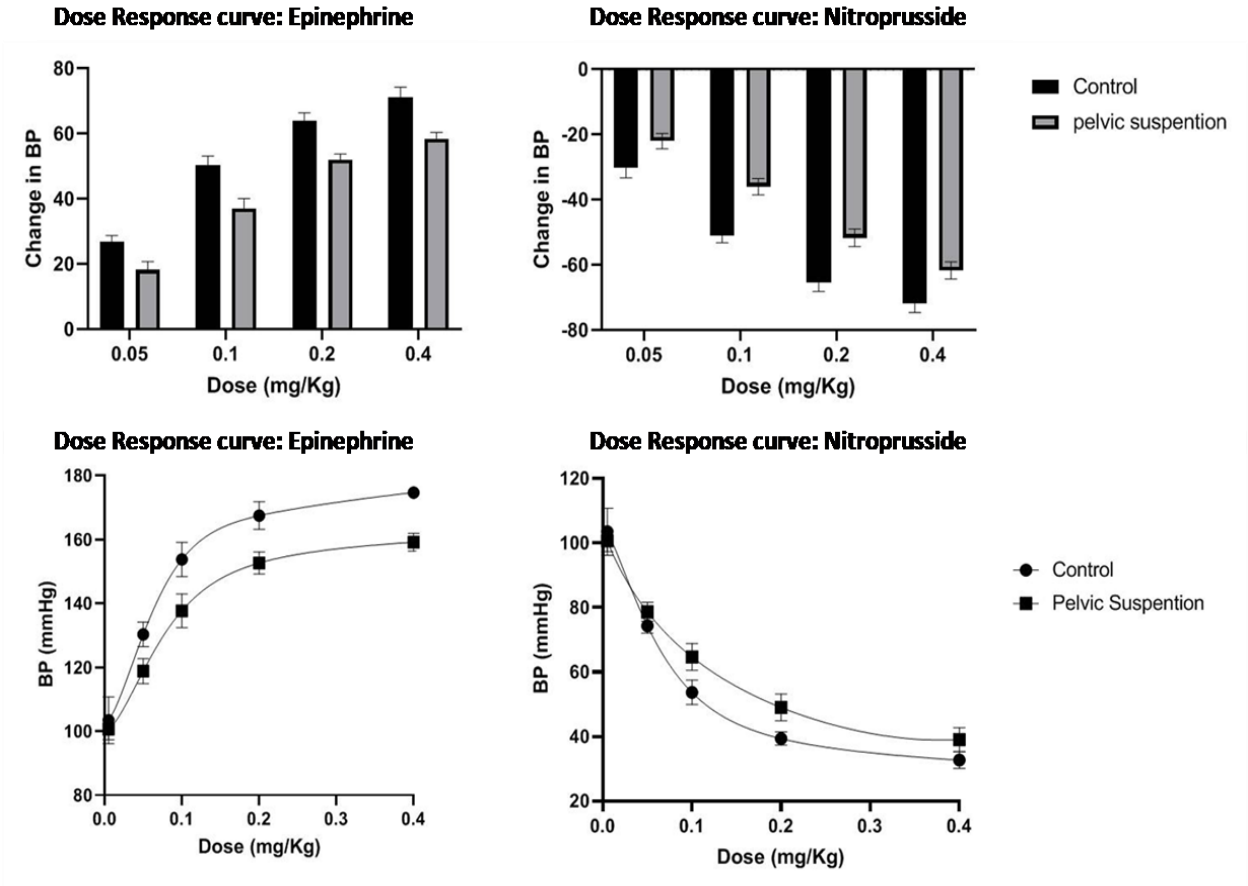

Figure 5: Dose response curve for different dose of epinephrine and nitroprusside

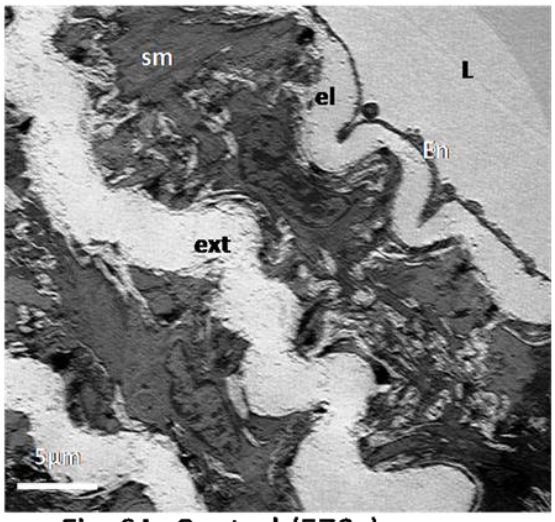

Fig. 6A: Control (570x)

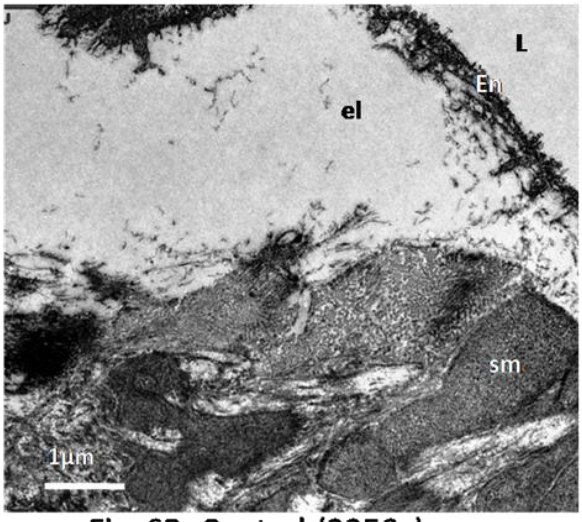

Fig. 6B: Control (2250x)

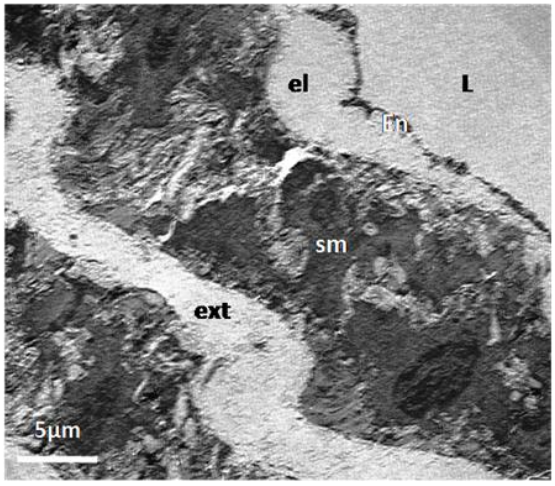

Fig. 6C: HU (570x)

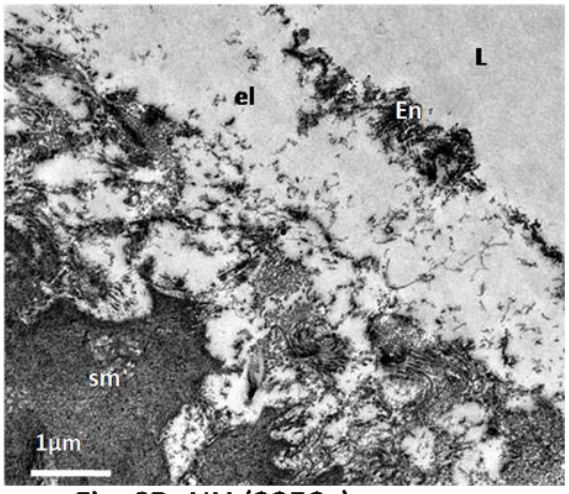

Fig. 6D: HU (2250x)

Fig 6: Electron microscopy of carotid artery ultra structure in different magnifications. Lu (Lumen), En (endothelial layer), el (internal elastic lamina), sm (smooth muscle), ext (external elastic lamina),

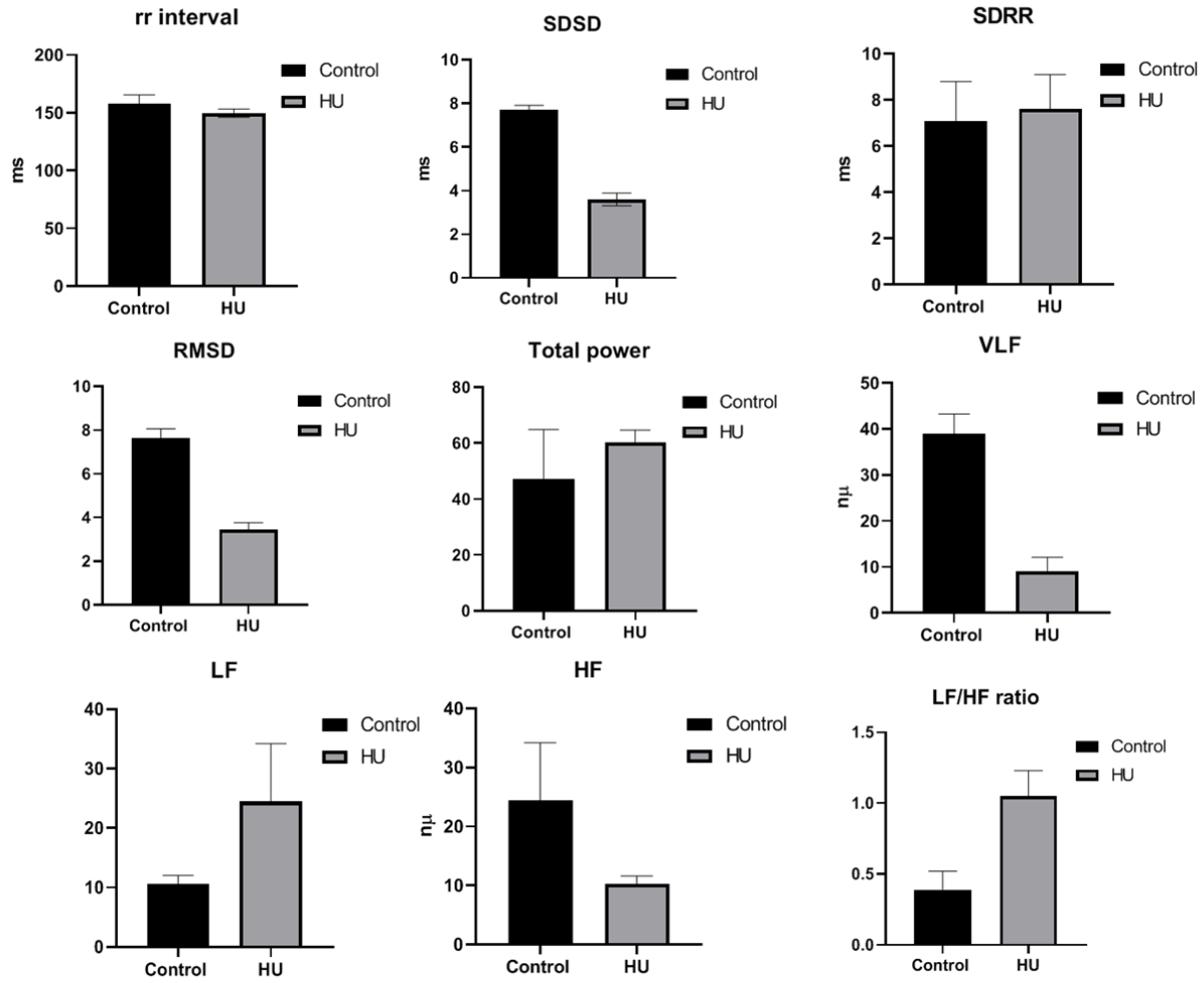

Fig 7: Comparison of Heart rate variability between control and HU rats