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HISTOLOGICAL CHANGES IN THE HEART AND THE PROXIMAL AORTA IN EXPERIMENTAL DIABETIC RATS FED WITH *PIPER SARMENTSOUM*

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Abstract

Cardiovascular complications are one of the major causes of death in diabetes mellitus. *Piper sarmentosum* (*P.s*) is an herb that possesses antihyperglycaemic effects. The main aim of the study was to observe the histological changes in the heart and the proximal aorta of streptozotocin-induced diabetic rats following *P.s* administration. Twenty-four male Sprague-Dawley rats (n=24) were equally randomized into four groups: control group supplemented with normal saline (C); control group supplemented with *P.s* (CTx) ; diabetic group supplemented with normal saline (D) and, diabetic group supplemented with *P.s* (DTx*).* Diabetes was induced by STZ (50mg/kg body weight) intramuscularly. *P.s* extract (0.125g/kg) was administered orally for 28 days, following four weeks of STZ induction. The cardiac and aortic tissues were collected and processed under different stains: Haematoxylin and Eosin (H & E), Verhoeff-Van Gieson (VVG), Masson's Trichome (MT) and Periodic Acid- Schiff (PAS). There were abnormal cardiomyocytes nuclei, disarray of myofibres and increase in connective tissue deposits in cardiac tissues of the diabetic untreated group. The thickness of tunica media and ratio of tunica intima to media were found to be significantly increased in the aorta of diabetic untreated group ($P < 0.05$) compared to the control group. There were degenerative changes in the proximal aorta in diabetic untreated groups. All the histological damages of cardiac and aortic tissues were found to be lesser in the diabetic treated groups. Supplementation with *P.s* extract prevented the oxidative damage arising from diabetes mellitus, and reduced its complications.

Keywords: *Piper sarmentosum*, diabetes mellitus, heart, aorta, histology.

Introduction

Diabetes Mellitus (DM) is a controllable metabolic disorder that continues to be a global health problem in the developed as well as developing nations. Epidemiological studies showed that diabetes itself promoted cardiac dysfunction directly or premature atherosclerosis, indirectly (Kawaguchi et al., 1997). According to Framingham study, the risk factors and incidences of developing heart failure increases in DM regardless of hypertension, obesity, hyperlipidemia and underlying coronary heart disease (Kannel et al., 1974). Various studies reported the cardiovascular complications in DM by using human and animal subjects.

It has been well understood that the endomyocardial fibrosis, direct toxic effect of hyperglycaemia on cardiomyocytes, endothelial dysfunction and oxidation of low density lipoprotein could play a crucial role in both type 1 (insulin deficient) and type 2 DM (insulin resistant). The characteristic features like structural and metabolic damage of cardiomyocytes in the cardiac tissue and increase foam cell formation with smooth muscle cell proliferation in the major blood vessels like aorta, have been reported by past researchers (Tzikas et al., 2005).

The relationship between the oxidative stress and DM is well defined. Previous studies concluded that increased oxidative stress is found in both type 1 and type 2 DM. Oxidative stress in DM results in an increase in the formation of free radicals and decrease in antioxidant potential (Nazirogjlu et al., 2005). Glucose autoxidation, cellular oxidation and interaction of elevated blood glucose with the long-lived protein in blood vessels wall contributes to the increase in oxidative stress formation in DM (Penckofer et al., 2002). The glycosylation of long-lived proteins can yield very stable complex products known as advanced glycosylation end-products (AGEs). The accumulation of these AGEs increase resistance to proteolysis and results in fibrosis (Aronson, 2003). Furthermore, the AGEs play important roles in cell signalling by interacting with specific cellular receptors called AGE receptors (RAGE). Thus, these receptors accelerate to enhance the atherosclerosis formation by activating the adhesion molecules, pro-inflammatory cytokines and growth factors (Schmidt et al., 1999).

Data from both scientific and laboratory studies show that herbs containing high levels and strong antioxidant compounds could modify the oxidative stress disorder such as DM rather than synthetic ones (Chanwitheesuk et al., 2005). Malaysia is a country with plenty of medicinal herbs and notable amongst them is *Piper sarmentosum* (*P.s*). In

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Malaysia, *P.s* is locally known as 'daun kadok' and it is used for treating minor ailments such as headache, joint ache, waist pain and menstrual pain (Subramanium et al., 2003).

Biochemically, *P.s* is known to possess various active compounds such as alkaloids, amides, flavonoids, phenylpropanoids. Laboratory studies showed that *P.s* possessed high antioxidant efficacy as it showed 87.6% of superoxide free radical scavenging activity, 98% of lipid peroxidation inhibitory activity and 96% of radical scavenging activity (Subramanium et al., 2003). The flavonoid compounds like Quercetin have been found to improve the endothelial dysfunction and Narigenin is proven to possess high superoxide scavenging activity (Vessal et al., 2003). Moreover, Tannins could increase the secretion of insulin and reduce hyperglycaemia in experimentally induced diabetic rats (Latte and Kolodziej, 2004).

It has been reported that *P.s* possesses antioxidative, antihyperglycaemic, antiatherogenic, anti-inflammatory, anticarcinogenic properties and hypoglycaemic properties. Past studies revealed that repeated administration of aqueous extract of the whole plant *P.s* has hypoglycaemic effect on normal and streptozotocin-induced diabetic rats (Peungvicha et al., 1998). The crude extracts of *P.s* reduced the blood-glucose levels in the alloxan-induced diabetic rabbits (Pongmarutai, 1989). Eventually, the administration of antioxidant compounds could modify DM and its complications. Thus, the main aim of the present study was to observe the effect of aqueous extract of *P.s* on the microscopic anatomy of the heart and the proximal aorta in the streptozotocin-induced diabetic rats treated with or without P.s extract.

Materials and Methods

Preparation of aqueous P.s extract

Fresh *P.s* leaves (5kg) were obtained from Mentah Resources, Negeri Sembilan, Malaysia and the plant was identified by a plant botanist from Universiti Kebangsaan Malaysia with the Voucher Specimen – UKMB (No. 29851). The *P.s* leaves were oven dried at 50°C and grounded to obtain *P.s* powder. Then it was mixed with 1 L of boiling water for 1 hour. The mixture was then filtered and the filtrate was sent to FRIM (Forest Research Institute Malaysia) to obtain the freeze-dried powder form. The freeze-dried powder extract was stored in dark bottles and kept in 4°C, until used. The *P.s* powder was dissolved in the normal saline and administered orally in the dose of 0.125g/kg body weight (Amran et al., 2010).

Animals and study design

A total of 24 adult male Sprague-Dawley rats weighing 200-250 gm were obtained from Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia. Prior ethical approval was obtained from the Institutional Animal Ethics Committee. All the rats were housed in isolated cages under air-conditioned room with adequate ventilation, a 12-hrs light/dark cycle and had free access to water and rat chow throughout the study period. All the rats underwent an acclimatization period for 1 week before the commencement of the study. The animals were then equally divided into two main groups: control group (n=12) and experimental group (n=12). The experimental group received single dose 50mg/kg body weight of streptozotocin (STZ) (SIGMA, ST. LOUIS, MO) injection intramuscularly. However, control group were injected with an equal amount of normal saline intramuscularly. Three days following STZ induction, the fasting blood-glucose level was measured from the tail vein using Accu-Check Advantage glucometer (BOEHRINGER MANNHEIM CORPORATION, INDIA-NAPOLIS, IN). Any fasting blood -glucose level greater than 8mmol/L was labelled as diabetic (Teoh et al., 2009). Following 4 weeks of STZ induction, both groups of rats were further sub-divided into two groups each: control group supplemented with normal saline vehicle (C group) (n=6), control group supplemented with 0.125g/kg of *P.s* extract (CTx group) (n=6), diabetic group supplemented with normal saline vehicle (D group) (n=6) and diabetic group supplemented with 0.125g/kg of *P.s* extract (DTx group) (n=6). The treatment in all the groups was continued for 28 days following STZ induction. Following 28 days of treatment, all the rats were sacrificed with high dose of diethyl ether. The thoracic cavity was opened and the heart and the proximal aorta (approximately 2 cm long) were dissected. The adherent adipose tissue and connective tissue were cleaned. For the histological examination, the cardiac tissue was dissected longitudinally and the aortic tissue was dissected transversely.

Histological study

For the light microscopic study, the specimens were fixed in 10% formaldehyde, underwent a dehydration process with alcohol series, embedded in paraffin wax using the tissue-embedding centre (LEICA EG 1160, WETZLAR, Germany). Tissues were sectioned (3-5 sections/specimen) using a microtome (MICROM HM 340E, WALLDORF, Germany) with 5µm thin sections. For the cardiac tissue, the specimens were stained with standard staining like Haematoxylin and Eosin (H&E), Verhoeff-Van Gienson (VVG) to observe the elastic fibres and Masson's

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Trichome (MT) staining to observe connective tissue deposits. For the proximal aortic tissue, the specimens were stained with H&E, VVG and PAS stains to find out the glycogen deposits. Four measures per image were obtained at 0, 90°, 180°, and 270° to estimate the thickness of tunica intima (TI) and tunica media (TM) (Moraes-Teixeira et al., 2010). The ratio between TI and TM was also calculated. The histological examination was carried out at a magnification of x200 for aorta and x400 for the cardiac tissue using Pix-elink colour camera (USA) with a computerized image analysis system Video Test T-morphology 5.1 software with a light microscope (LEICA DM RXA2, Germany).

Statistical analysis

The data was analyzed by using two-way ANOVA followed by a Bonferroni test. Continuous variables were shown as mean ± SD and a value of $P < 0.05$ was considered significant. All statistical analysis was performed by using SPSS statistical version 19 (SPSS Inc., USA).

Results

Histology of the cardiac tissue

Under H&E and VVG staining, C group showed single, oval and centrally located nuclei of cardiomyocytes with regularly arranged cardiac myofibres (Figure 1 A, 2 A). A similar pattern could be found in CTx group (Figure 1 B, 2 B). However, nuclei of the cardiomyocytes in D group showed deformation in sizes and shapes. Moreover, the cardiac myofibres in this group was found to be in disarrayed pattern compared to C group (Figure 1 C, 2 C). This was probably due to the degeneration of the structural protein in mitochondria of the cytoplasm that occurred in protein degradation related to DM. DTx group was found to possess fewer severe histological changes in the cardiac tissues compared to the D group (Figure 1 D, 2 D) .Both C group and CTx group did not show any connective tissue deposits (Figure 3 A, B). Whereas, interstitial fibrosis was observed as connective tissue deposits (stained blue) in D group (Figure 3 C) compared to the C group. The connective tissue deposits appeared to be much less in DTx group (Figure 3 D) than in D group.

Table 1: Measurements of thickness of the tunica intima and tunica media of the aortic wall (n = 6) and their ratio.

Group	C	CTx	D	DTx
Thickness TI (µm)	4.09 ± 1.56	3.78 ± 1.43	5.91 ± 2.30#	3.67 ± 1.07#
Thickness TM (µm)	106.54 ± 6.91	98.39 ± 8.53	140.26 ± 19.02*	99.07 ± 12.37*
Ratio TI:TM	0.04 ± 0.01	0.04 ± 0.01	0.09 ± 0.04*	0.05 ± 0.03*

* Significant *P* value determined by a Bonferroni test ($P < 0.05$); # Non significant

Morphological findings

The morphological measurements of thickness of the tunica intima (TI), tunica media (TM) and the ratio of TI: TM was performed under H&E staining. C and CTx groups showed no significant changes in the thickness of TI and TM. On the other hand, significant increase in thickness of TM was observed in the D group compared to C group. In DTx group, the thickness of TM was significantly decreased ($P < 0.05$) compared to the D group (Table 1). However, the thickness of TI did not show significant differences between all the four groups ($P > 0.05$).
As shown in Table 1, the ratio of TI: TM was found to be significantly increased in D group compared to the C group. The ratio of TI: TM also changed significantly in DTx group ($P < 0.05$).

Histology of the aortic tissue

Under H&E staining, there was no histological difference in the C (Figure 4 A) and CTx (Figure 4 B) group. The increase thickness of TI and TM layers were observed in the D group compared to the C group. The thickness of TM was found to be more prominent than the thickness of TI (Figure 4 C). This was due to the proliferation of smooth muscle cells in the aortic wall. However, the degenerative changes were less severe in DTx group compared to the group D (Figure 4 D). The findings were also similar in PAS staining of the aorta, in which the D group was found to have prominent histological damages (Figure 6 C). The damages were less in DTx group (Figure 6 D) compared to the D group. No obvious damages of the aorta were observed in the C and CTx groups (Figure 6 A, B).. Under VVG staining, the disruption of elastic fibres in the tunica media layer was observed in the D group (Figure 5 C) than in the C and CTx groups (Figure 5 A, B). The arrangement of the elastic fibres in the DTx group showed less disruption compared to the group D (Figure 5 D).
All the histological results were analyzed by two expert observers in a double blinded manner, under image analysis software.

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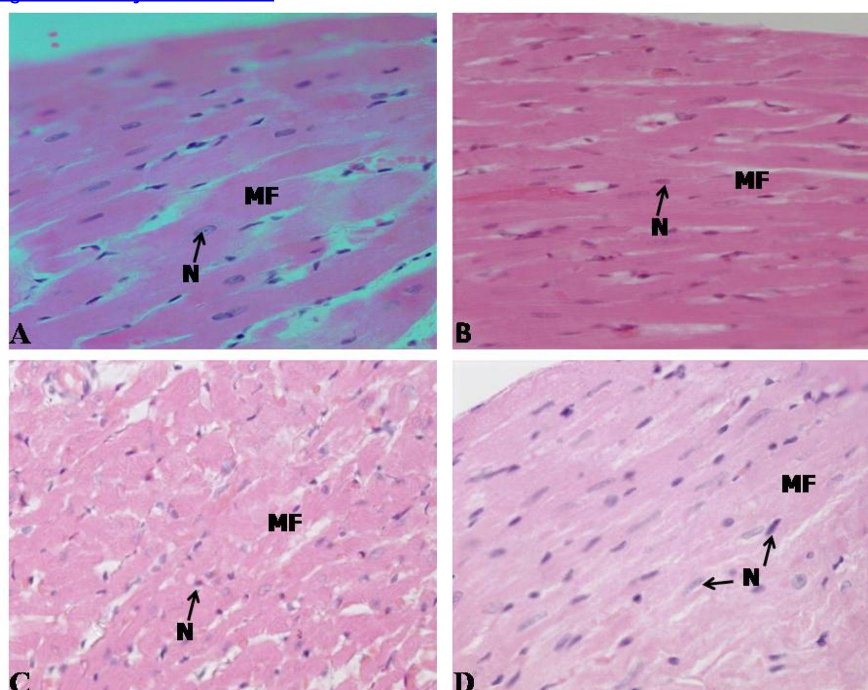

Figure 1: Photomicrograph showing longitudinal section of cardiac tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* **(C)** diabetic group supplemented with normal saline and **(D)** diabetic group treated with 0.125g/kg ***P.s* extract** where **N**: nuclei of cardiomyocytes, **MF**: myofibres under Haematoxylin and Eosin stain. (LM x400).

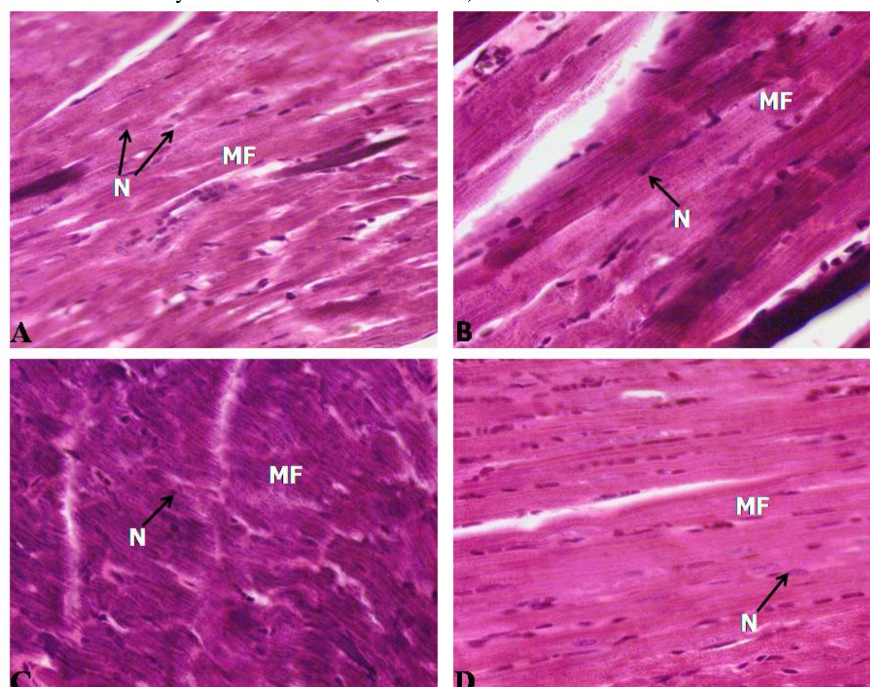

Figure 2: Photomicrograph showing the longitudinal section of cardiac tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* extract **(C)** diabetic group supplemented with normal saline and **(D)** diabetic group treated with 0.125g/kg where **N**: nuclei of cardiomyocytes, **MF**: myofibres under Verhoeff-Van Gieson stain. (LM x400).

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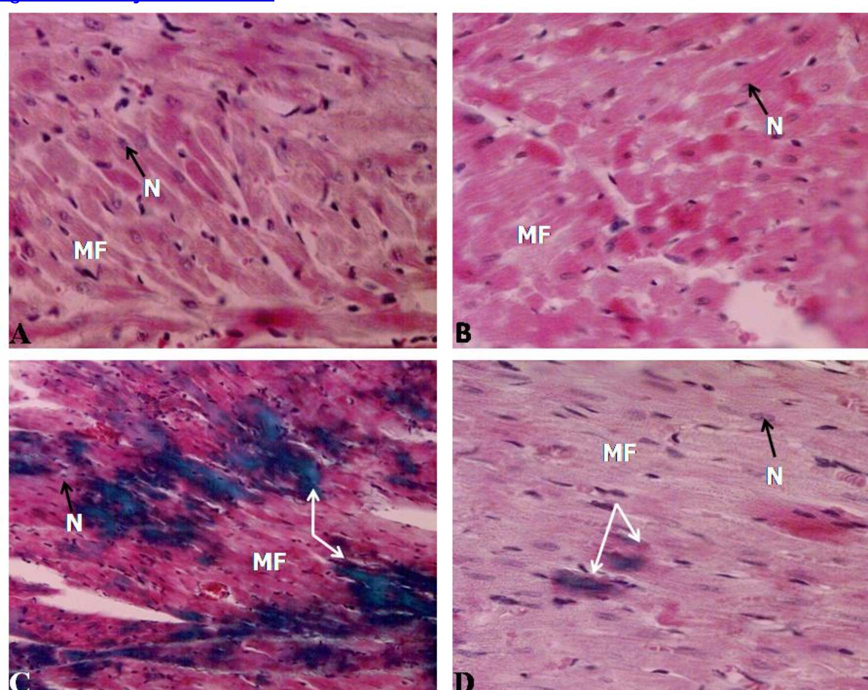

Figure 3: Photomicrograph showing the longitudinal section of cardiac tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* extract **(C)** diabetic group supplemented with normal saline and **(D)** diabetic group treated with 0.125g/kg where **N:** nuclei of cardiomyocytes, **MF**: myofibres, white arrows: connective tissue under Masson's Trichome stain. (LM x400).

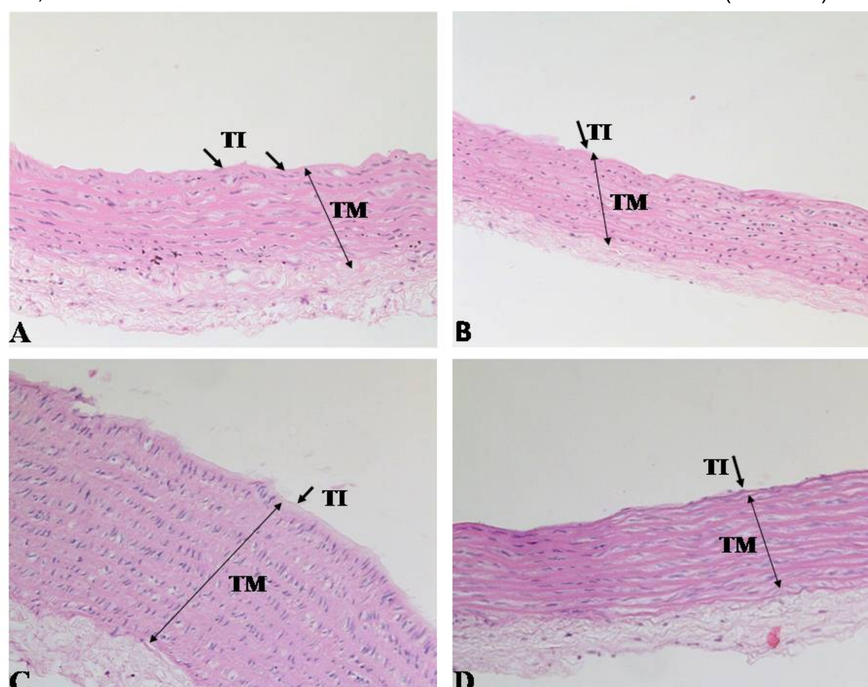

Figure 4: Photomicrograph showing the section of aortic tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* extract **(C)** diabetic group supplemented with normal saline and **(D)** diabetic group treated with 0.125g/kg where **TI**: tunica intima, **TM**: tunica media, Black arrows: endothelial cells under Haematoxylin and Eosin stain. (LM x200).

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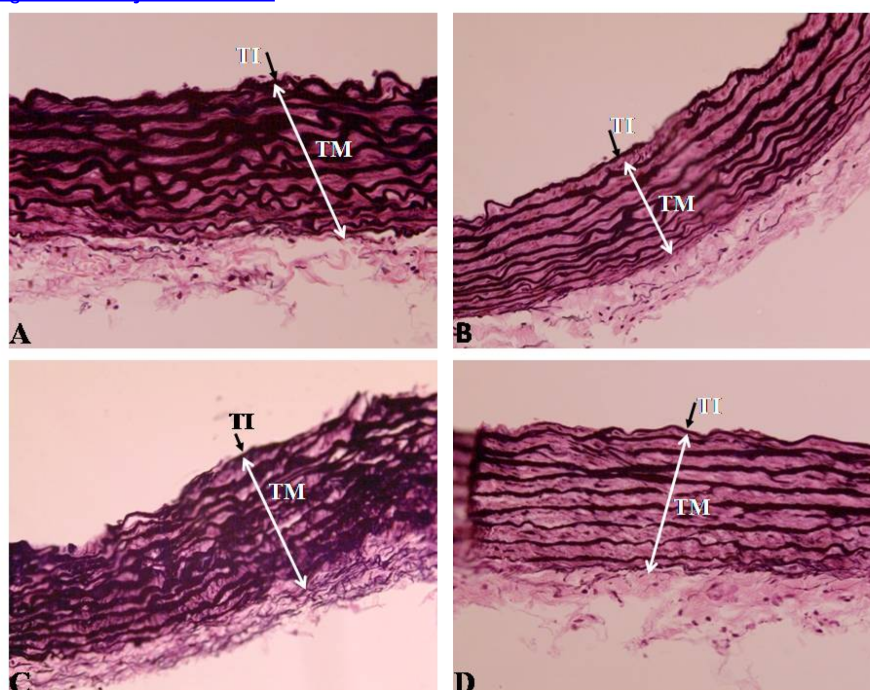

Figure 5: Photomicrograph showing the section of aortic tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* extract **(C)** diabetic group supplemented with normal saline and **(D)** diabetic group treated with 0.125g/kg where **TI:** tunica intima, **TM:** tunica media, Black arrows= endothelial cells under Verhoeff-Van Gieson stain. (LM x200).

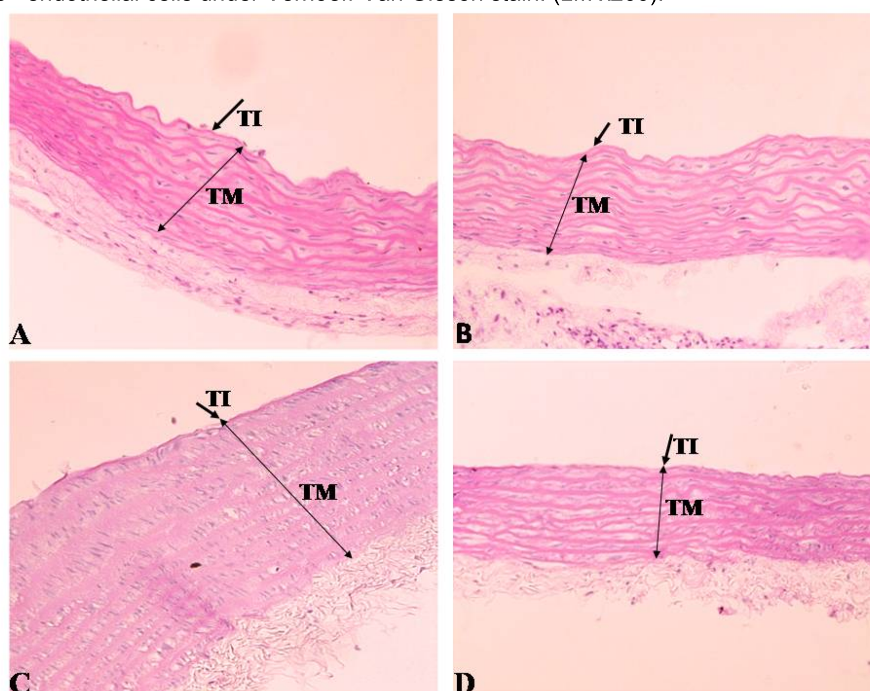

Figure 6: Photomicrograph showing the section of aortic tissues in **(A)** control group supplemented with normal saline **(B)** control group supplemented with 0.125g/kg *P.s* extract **(C)** diabetic group supplemented with normal

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saline and **(D)** diabetic group treated with 0.125g/kg where **TI:** tunica intima, **TM:** tunica media, Black arrows= endothelial cells under Periodic Acid-Schiff stain. (LM x200).

Discussion

In the present study, the STZ-induced diabetic rats are used as a type 1 models which are characterized by the uncontrolled hyperglycaemia, hypoinsulinemia and ketoacidosis (Nemoto et al., 2006). It was estimated that approximately 30% of patients with type 1 diabetes may develop a variety of complications (Soltani et al., 2007). STZ induced rats serve as excellent experimental animal models of DM. It is suitable for studying the basic mechanisms of diabetic cardiovascular complications and their time -dependent progression (Pieper, 1999). In the control group of rats, the nuclei of the cardiomyocytes were single, oval, prominent and centrally located in cardiomyocytes as observed under the light microscope. For the aortic tissues, it revealed the presence of the endothelial cells in intima layer, smooth muscle cells in the tunica media layer. It may be mentioned that the tunica intima layer has been reported to constitute one-fourth of the entire aortic wall, with the media layer being the thickest layer (Aymen, 2000).
The histological findings of the cardiac tissue in the present study showed that structural organization of cardiac tissues was disturbed in STZ-induced diabetic rats. Inflammatory histological changes in cardiac tissues indicated the myocardial injury. Deformation of nuclei of cardiomyocytes and disarrangement or disordered cardiac myofibres was observed under H & E staining and VVG staining. In fact, with MT staining, the connective tissue deposits (shown with blue stain in Figure 3 C) were observed in the group D. These findings were considered important for the development of diabetic cardiomyopathy.

Previous studies reported diabetic cardiomyopathy characterized with systolic or diastolic dysfunction and cardiac fibrosis in diabetic patients (Hayat et al., 2004). The present finding in the connective tissue deposit under MT staining was similar to past research studies. In diabetic cardiomyopathy, the increase in heart weight, increase in sizes of cardiomyocytes nuclei of type 2 diabetic rats were reported by past researchers. However, in our study, the changes in shapes and sizes of nuclei of cardiomyocytes with disarrayed cardiac myofibres were observed in type 1 diabetic rats under H&E and VVG staining. These findings were more likely towards the cardiac atrophy with a decrease in the cardiomyocytes, which were features of STZ-induced diabetic cardiac dysfunction. These findings were also reported in an earlier study (Cosyns et al., 2007). The cellular mechanism involved in type 1 diabetes is not clearly understood. To the best of our knowledge, in the state of insulin deficiency (type 1 DM), there was a decrease in the protein synthesis, increase in the protein degradation, defective mitochondrial function with loss of myofibrils in the myocardium (Nemoto et al., 2006) and this might induce disruption in the cardiac myofibres. In addition, hyperosmolarity in hyperglycaemia, shrinking of ventricular cardiomyocytes, increase of collagen bundles in type 1 DM, may result in changes in the size and shape of the nuclei of cardiomyocytes (Malone et al., 1999).

In the present study, the findings in the cardiac tissues were found to be lesser in DTx group compared to the D group. *P.s* herb is enriched with anti-oxidant compounds like flavonoids, vitamin C and E (Subramanium et al., 2003). The antioxidant activity of the *P.s* extract, which occur through its free-radical scavenging activity, may have prevented the oxidative damage at the myocardium in STZ-induced diabetic rats. Furthermore, the antioxidant activity of quercetin contributed in *P.s* extract would be helpful to manage glucose uptake and the glucose-induce increased levels of mitochondrial reactive oxygen species (ROS) linked to hyperglycaemia (Jo et al., 2009). The results of the present study showed that treatment with *P.s* extract decreased the cardiac damage in STZ-induced diabetic rats.
Prolong hyperglycaemia itself could induce the development of premature atherosclerotic lesions. Proliferation of the medial smooth muscle cells were observed in the aortic wall. According to the present findings, a significant increase in the thickness of tunica media was observed in STZ-induced diabetic rats. These findings are consistent with the earlier work which mentioned the smooth muscle cells proliferation in the tunica intima and tunica media layer of the ascending aorta (Balkis et al., 2009). The underlying mechanisms between DM and premature atherosclerosis still remain unclear. Hence, it was suggested advanced glycosylation end products (AGEs) interact with RAGE and the oxidative stress results in the increased production of free-radicals (ROS). These AGEs can promote the atherosclerotic process by enhancing the oxidation of low-density lipoprotein (LDL) mediated by RAGE. Thereby, macrophages with foam cells formation in the tunica intima layer and smooth muscle cells proliferation in medial layer were observed in diabetic atherosclerosis (Aronson and Rayfield, 2002). However, in the present study, tunica intima thickness did not show any significant changes compared to the control group. This might be due to the less effect of DM disease on foam cell formation in the tunica intima layer of the aortic wall rather than cholesterol induced atherosclerosis.

The histological findings on the aortic wall even demonstrated that there was an increase in the medial thickness in the proximal aorta in the D groups when viewed under H&E and PAS staining. Under VVG staining, the elastic fibres were found to be disordered in the D group than in the DTx group. According to the present findings, it was proven that *P.s* extract had a protective role on atherosclerotic lesion. *P.s* extract possessed the compound 'narigenin' which has anti-atherogenic effect (Aronson and Rayfield, 2002). These flavonoid compounds could reduce

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the risk of developing coronary heart disease by decreasing the LDL oxidation (Seong et al., 2001). These are the important steps in inhibition of the pathogenesis of the atherosclerosis.

Conclusion

In conclusion, treatment with *P.s* extract had a beneficial effect on experimentally induced diabetic rats. Treatment with *P.s* extract maintained the histological integrity of the cardiac tissues by reducing the degenerative changes in the myocardium as well as in the aortic tissues by decreasing the thickness of tunica media. These modified DM and its complications. Further studies are needed to corroborate such facts.

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