

Uncontrolled Diabetes with Underlying Structural Changes in the Atrioventricular Node

Diabetes no Controlada con Cambios Estructurales Subyacentes en el Nodo Atrioventricular

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SUMMARY: Diabetic cardiomyopathy, characterized by diabetes mellitus (DM) -induced cardiac muscular abnormalities, is a strong inducer of impaired cardiac contraction and arrhythmia. Atrioventricular block, a serious type of arrhythmia resulting from interruption of cardiac impulse conduction via the atrioventricular node (AVN), frequently occurs among diabetic patients. However, details of structural changes in AVN in DM remain poorly explained. Here, this study defined the effects of DM on the morphological remodeling of the AVN in male Sprague Dawley rats induced by intraperitoneal injection of streptozotocin (60 mg/kg body weight). At 24 weeks, the pathological changes in the AVN were assessed by light microscopy (LM) and transmission electron microscopy (TEM). Under LM, the AVN in diabetic rats became a less compact mass and exhibited the intracellular vacuolation. The nodal cells were more varied in sizes with the absence or shrinkage of nuclei and clear cytoplasm compared to the control. The collagen content significantly increased in relation to the presence of myofibroblasts. Consistent with LM, TEM images of the diabetic nodal cells revealed several signs of cell damage, such as mitochondrial changes, deterioration of cell organelles, gap junction internalization, and cell separation. Furthermore, changes in AVN innervation, evidenced by damaged Schwann cells and axons, were also found. These results indicated alterations in important components in the AVN during diabetic condition, which may lead to the impairment of electrical conduction, causing abnormal cardiac functions in diabetic patients.

KEYWORDS: Diabetes mellitus, Arrhythmia, Atrioventricular node.

INTRODUCTION

Inadequate glycemic control over a long period of time in diabetic conditions is associated with many complications and death in patients with type 1 and type 2 diabetes mellitus (DM) (Shi *et al.*, 2021). Diabetic cardiomyopathy, a common diabetic complication related to cardiac structural and functional abnormalities, impairs not only the contractile part but also the electrical conducting part of the myocardium, causing arrhythmia. The atrioventricular node (AVN), which is an electrical gatekeeper between the atrium and ventricle, is the common potential point of arrhythmogenesis, known as atrioventricular (AV) block. DM is significantly associated with an increased incidence of complete AV block, which completely impedes action potential (AP) impulse conduction from the atrium to the ventricle. Then, the patients experience bradycardia, cardiogenic shock, arrest, sudden death, and the clinical condition warranting permanent pacemaker implantation (Movahed *et al.*, 2005). Previous

evidence has shown electrophysiological remodeling in AVN myocytes of streptozotocin (STZ) rats, including an increase in AP duration and a slow spontaneous AP rate and upstroke velocity. In accordance with AP changes, the hyperpolarization-activated current, L-type calcium current, and delayed rectifier potassium current are reduced in DM, which affect AP generation in slow depolarization, rapid depolarization, and repolarization, respectively (Yuill *et al.*, 2010). Interestingly, the AVN in diabetic patients is upregulated by various genes, relevant to membrane ion channels (such as Hcn3, Slc8a1, Kcnh 2) and calcium homeostasis (Ryr2,3), which are suggested to be a compensatory mechanism to restore the ionic current flow of the AVN (Howarth *et al.*, 2017). Moreover, the apoptotic and necrotic cell death of AVN myocytes are identified in autopsy hearts from patients with type 2 DM (Hostiuc *et al.*, 2013). Although there are strong evidences, that DM is the

underlying cause of AVN electrophysiological changes, the cellular pathology in AVN myocytes has been poorly explored. Therefore, this study aimed to provide further insight into the effects of diabetes on the development of AVN abnormalities in terms of histopathological and ultrastructural details. Light microscopy (LM) and transmission electron microscopy (TEM) were used to study AVN in the diabetic rat model, compared to the age-matched control group. This knowledge can be useful for predicting the development of arrhythmia and potential therapeutic strategies to prevent lethal cardiac events in diabetic patients.

MATERIAL AND METHOD

Experimental animals and diabetic induction. Eighteen male Sprague Dawley rats (5-8 weeks old, 200-270 g body weight) were used in accordance with the Guide for the Care and Use of Laboratory Animals. All laboratory protocols were approved by the Siriraj Animal Care and Use Committee, Mahidol University, Thailand (COA No. 008/2561). The rats were divided into DM (n= 10) and control groups (n= 8). The diabetic induction method using STZ was performed following a previous study (Lerkdumnerkit *et al.*, 2022). At 24 weeks after induction, the rats were sacrificed by halothane inhalation and then processed for LM (DM; n=5, control; n=4) and TEM. (DM; n=5, control; n=4).

Tissue preparations for LM and TEM. After sacrifice, the hearts were removed and cut at the atrioventricular septa, which contained the AVN. In the LM, the septa were preserved in Bouin's solution and then conventional histological processing with Masson's trichrome staining was performed. For TEM, the septa were fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffered saline. The septa were processed into plastic-embedded tissue (Chookliang *et al.*, 2021). The ultrathin sections were cut at 70 nm thick for uranyl acetate and lead citrate staining. Ultrastructural observation of AVN was performed under a Philips Tecnai F20 TEM. (Hillsboro, Oregon, USA).

Table I. Glycemic parameters and body weights of control and DM groups.

Parameters	Groups	
	Control	Diabetes
Blood glucose (mg/dL)	122.00 ± 11.19	451.62±22.72 ^a
Urine glucose (mg/dL)	0	≥500 ^b
Body weight (g)	405.20±38.56	239±13.86*

Blood glucose and body weight levels were showed as mean± SD. a. Diagnostic value of diabetes (≥300 mg/dL of fasting blood glucose, ≥500 mg/dL of urine glucose). **p-value ≤0.05 diabetes versus control

Collagen analysis. To determine the AVN collagen content, 20 random sections of AVN in each group were photographed under 400× magnification. The percentage of collagen area per total designated area (175 μm × 130 μm) was calculated as collagen volume fraction by using imageJ software. The collagen volume fraction of AVN in each group was averaged and presented as mean ± standard error of the mean (SEM).

Statistical Analysis. The body weights of the rats in both the control and DM groups were compared between groups by using the Mann-Whiney U test, while individual group comparisons of the mean collagen volume fraction were correlated by using Student's t test. Statistical significance was considered at a p-value of 0.05 or less. All data were analyzed by using PASW statistics software.

RESULTS

Within approximately 48-72 hours after STZ injection, DM was diagnosed by high glucose levels in blood (above 300 mg/dL) and urine (above 500 mg/dL). Before sacrifice, the diabetic rats had a significant loss of the mean body weight, compared to control rats (Table I).

Histopathological analysis of AVN in diabetic rats. The low power microphotographs demonstrated an AVN mass at the base of the interatrial septum on the right side of the central fibrous body (Figs. 1A, 1B). The AVN presented as an oval shaped pale- stained lump that was easily distinguishable from the surrounding ordinary contractile myocardium of the atrium and ventricle (Figs. 1A-D). The compact AVN of control rat was made up of tightly packed nodal cells (Fig. 1C). This specialized cell was an irregular- or short spindle-shaped cell, arranged in multiple orientations, an oval-shaped nucleus with a heterogeneously lightly stained cytoplasm, and darker staining around the cytoplasmic margin. Among the nodal cells, prominent rod to elongated nuclei of interstitial fibroblasts were also found (Fig. 1E). At 24 weeks after diabetic induction, the AVN became a less compact mass and exhibited intracellular vacuolation (Fig. 1D). This characteristic resulted from an enlargement of the various sizes of nodal cells with a large volume of clear cytoplasm. Their nuclei were smaller and more irregularly shaped than those of the control; however, nuclei in some nodal cells were invisible. Additionally, active interstitial fibroblasts or myofibroblasts, characterized by a rounder- shaped and paler-stained nucleus with amorphous fibrillary cytoplasm, were notably found among the green field of collagen accumulation. Furthermore, the collagen fibers in the AVN became more densely packed in the diabetic group than in the control group (Figs. 1E, 1F). Consistent with histological features, the quantitative analysis of AVN collagen

contents showed a significant increase in diabetic rats (5.143 ± 0.255), compared to control rats (0.6156 ± 0.096) (Fig. 1G).

These results implied that DM induced AVN structural remodeling.

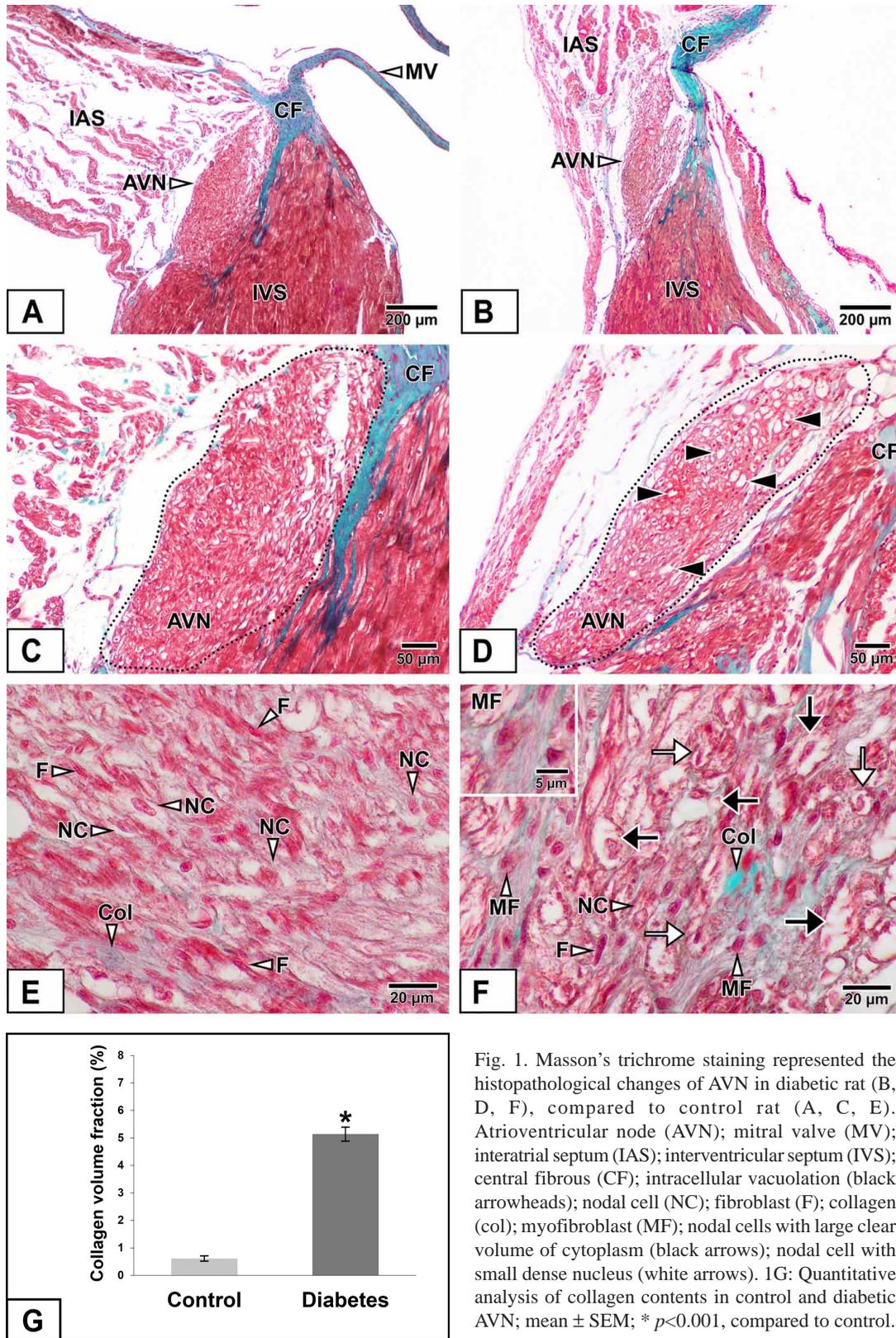


Fig. 1. Masson's trichrome staining represented the histopathological changes of AVN in diabetic rat (B, D, F), compared to control rat (A, C, E). Atrioventricular node (AVN); mitral valve (MV); interatrial septum (IAS); interventricular septum (IVS); central fibrous (CF); intracellular vacuolation (black arrowheads); nodal cell (NC); fibroblast (F); collagen (col); myofibroblast (MF); nodal cells with large clear volume of cytoplasm (black arrows); nodal cell with small dense nucleus (white arrows). 1G: Quantitative analysis of collagen contents in control and diabetic AVN; mean \pm SEM; * $p < 0.001$, compared to control.

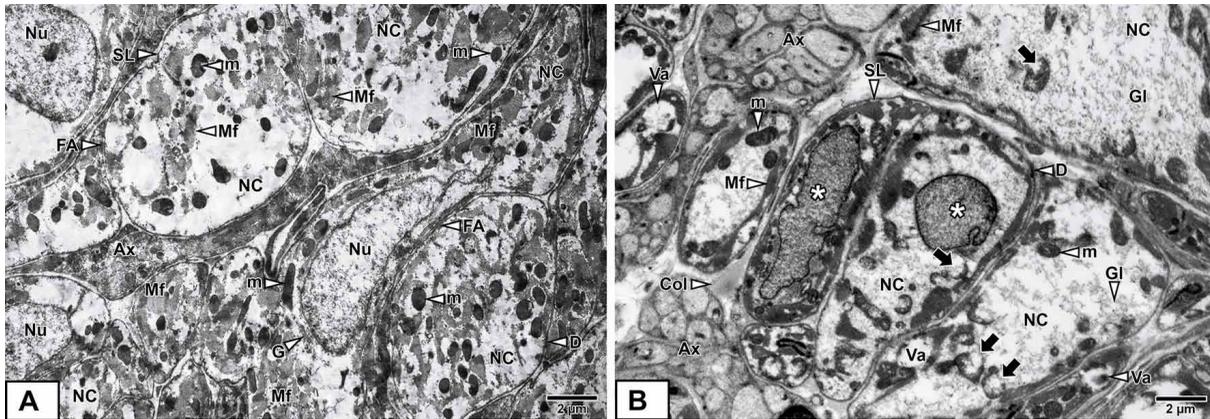


Fig. 2. TEM micrographs of AVN in the control (A) and diabetic (B) rats. Nodal cell (NC); nucleus (Nu); mitochondria (m); myofilament (Mf); Golgi apparatus (G); sarcolemma (SL); fascia adherens (FA); desmosome (D); axon (Ax); mitochondrial disruption (thick black arrows); shrunken nucleus with a higher electron-dense stain (white asterisks); glycogen (Gl); autophagic vacuole (Va); collagen fiber (col).

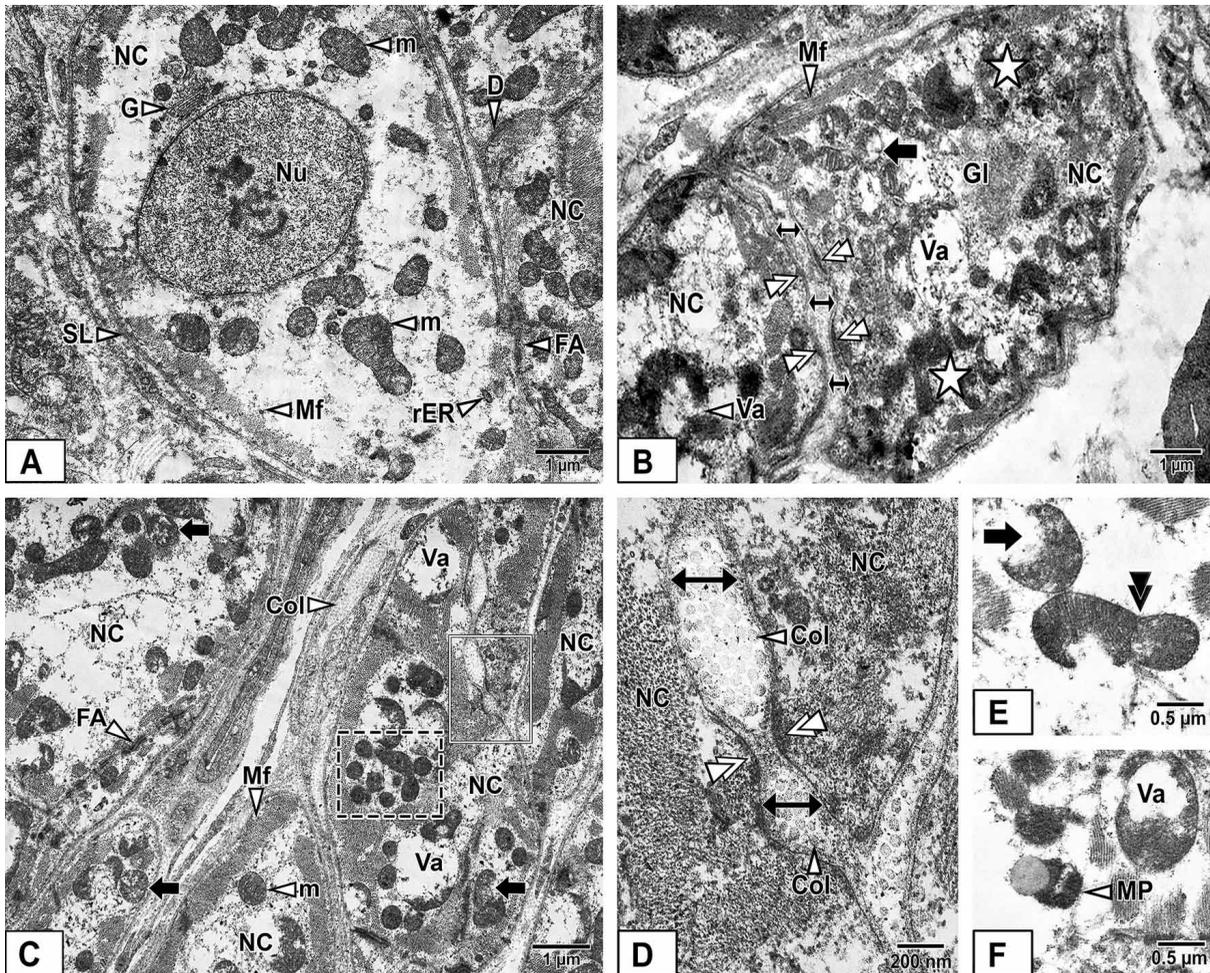


Fig. 3. Ultrastructural analysis of AVN in the control (A) and diabetic (B-F) rats. Nodal cell (NC); nucleus (Nu); mitochondria (m); myofilament (Mf); rough endoplasmic reticulum (rER); Golgi apparatus (G); sarcolemma (SL); desmosome (D); fascia adherens (FA); electron dense material (white stars); glycogen (Gl); mitochondrial disruption (thick black arrows); autophagic vacuole (Va); disrupted cell junctions (double white arrowheads); nodal cell separation (two-way arrows); collagen fiber (col); a group of small mitochondria (a black dashed square); mitochondrial fission (double black arrowhead); mitophagy (MP).

Changes in AVN ultrastructures in DM. Under TEM, the irregularly shaped nodal cells were enveloped by an electron dense layer of the unit membrane, called the sarcolemma. The oval nucleus was placed at the cell center and surrounded by several cytoplasmic organelles. Unlike contractile cardiomyocytes, myofilaments within nodal cells were poorly organized and scattered throughout cytoplasm, especially near the cell margin. Many elongated or round mitochondria were found as double membrane-bound organelles with abundant cristae. The flattened membrane-bound cisterna of the Golgi apparatus and rough endoplasmic reticulum (rER) were illustrated (Figs. 2A, 3A). In diabetic rats, several damaged characteristics of nodal cells in response to hyperglycemic stress were detected. The nodal nuclei shrank with higher electron-dense staining (Fig. 2B). Their cytoplasm presented a huge autophagic vacuole, characterized by a clear vacuole incorporated with electron dense indigestive materials. The cytoplasm of some nodal cells showed increased glycogen accumulation and disheveled electron dense debris, suggesting severe organelle deterioration in DM. The mitochondria were swollen, and their membrane and cristae were disrupted. Moreover, the diabetic nodal cells also presented mitochondrial fission, a large number of small dense mitochondria, and the formation of mitophagy, which was a fusion between damaged mitochondria with a homogenous and moderately dense vacuole of lysosome (Figs. 2B, 3B-C, 3E-F). As the nodal cells were in perfect contact with each other by intercellular protein bridges of cell junctions, including desmosomes, fascia adherens, and gap junctions (Figs. 3A, 4A), DM was able to disrupt cell junctions, leading to nodal cell separation. Interestingly,

interstitial collagen fibrils were interposed in the small area between two adjoining nodal cells (Figs. 3B-D). In addition, the formation of an annular profile of gap junctions, named connexosome, which was suggested as gap junction internalization, was identified (Fig. 4B). These were the leading causes of disrupted cell junctions and nodal cell separation. In terms of AVN innervation, the normal rat exhibited different sizes of myelinated and unmyelinated axons, inserted between the nodal cells. The myelinated axon was the axonal core, sheathed by a thick electron dense layer of myelin sheath, while several unmyelinated axons were embedded together within the Schwann cell pocket. Individual axons composed of neurofibrillary elements and small-round mitochondria. These axons were held together by thin connective tissue, the endoneurium (Fig. 5A). Compared with the control, the axons in AVN of diabetic rats contained mitochondrial swelling with fragmented cristae and clearer axoplasm, indicating axon swelling. Schwann cell wrapped around axon also presented autophagic vacuoles (Figs. 5B, 5C). Interestingly, a macrophage with cytoplasmic projection and abundant phagolysosomes inside was also seen in close contact with the damaged nerve within AVN in diabetic rat, indicating activated macrophage (Fig. 5D).

DISCUSSION

Diabetic cardiomyopathy affects not only contractile cardiomyocytes but also electrical cardiomyocytes. Because the pathological progression of both cardiac cell types is

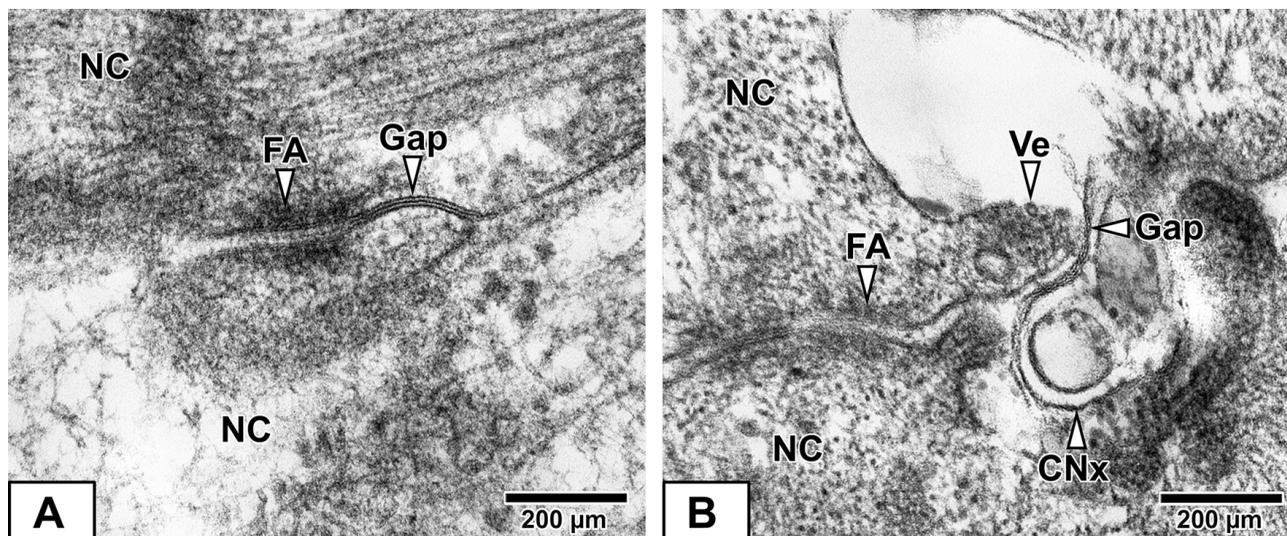


Fig. 4. TEM image of cell junctions between two nodal cells in the control (A) and diabetic (B) rats. Nodal cell (NC); gap junctions (Gap); fascia adherens (FA); the formation of connexosome (CNx); vesicle (Ve).

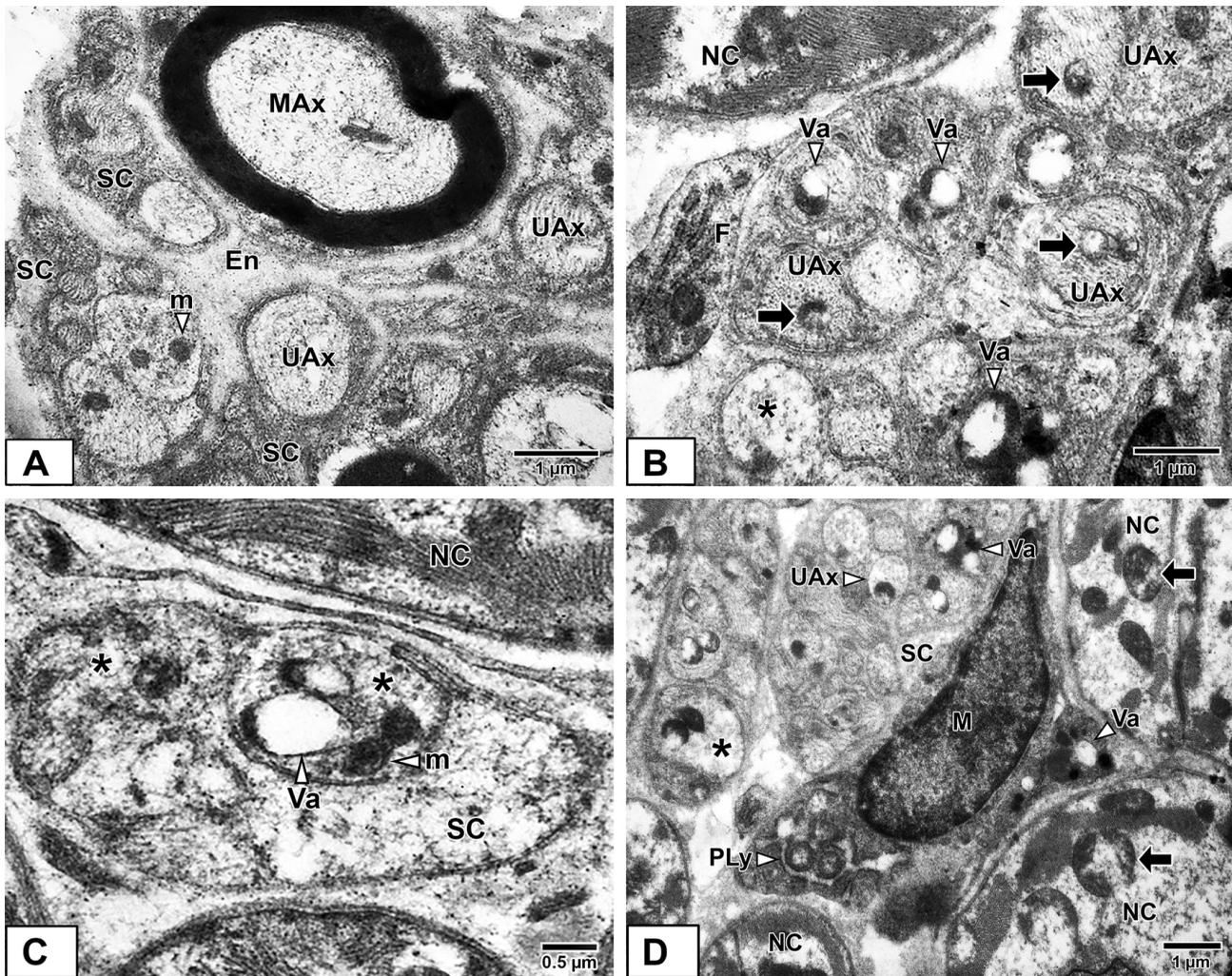


Fig. 5. Electron photomicrography represented the changes in AVN nerve innervation of diabetic (B, C, D), compared to control (A) rats. Myelinated axon (MAx); unmyelinated axon (UAx); mitochondria (m); endoneurium (En); Schwann cell (SC); nodal cell (NC); dilated mitochondria with disrupted cristae (black arrows); autophagic vacuole (Va); axonal swelling (black asterisks); fibroblast (F); macrophage (M); phagolysosome (PLy).

usually correlated, it is quite possible that diabetes might be involved in similar mechanisms to develop their pathology. DM is associated with electrical conducting disturbance at the AVN, which consists of electrical cardiomyocytes; likewise, altered AVN morphology in STZ-diabetic rats was found. In LM observation, the AVN nodal cells in diabetic rats varied in size and contained a large clear volume of cytoplasm, accompanied by the cytoplasmic clear area, a clump of electron dense materials, and autophagic vacuoles in the TEM images. These morphologies indicated nodal cell injury resulting from DM-induced osmotic stress and organelle deterioration, possibly by the polyol pathway, intracellular calcium ($[Ca^{2+}]_i$) dysregulation, and mitochondrial dysfunction. In the polyol pathway, excessive glucose in DM is reduced to sorbitol by aldose reductase.

The accumulation of intracellular sorbitol creates a higher osmotic pressure and water diffusion into the cytoplasm of cells, causing osmotic stress-induced cardiomyocyte injury. Moreover, the conversion of sorbitol to fructose by aldose dehydrogenase, generates reactive oxygen species (ROS), which cause oxidative damage to cellular components (Chung *et al.*, 2003; Burgos-Morón *et al.*, 2019). Thus, the polyol pathway might be associated with the increased cytoplasmic volume and deteriorated organelles in nodal cells in DM. In $[Ca^{2+}]_i$ dysregulation, the change in $[Ca^{2+}]_i$ homeostasis in diabetic hearts involves in the altered functions of SR Ca^{2+} -ATPase (SERCA) and ryanodine receptor (RyR), which are the Ca^{2+} regulatory proteins on the sarcoplasmic reticulum (SR) membrane. High glucose (HG) can lead to hyperactivated calcium/calmodulin-dependent

protein kinase II, which prevents cytosolic Ca^{2+} reuptake via SERCA and induces internal SR Ca^{2+} leakage via RYR, resulting in Ca^{2+} overload in cytoplasm (Hegyi *et al.*, 2021). A raised $[\text{Ca}^{2+}]_i$ causes the potential to activate calpain1, which is a Ca^{2+} -dependent protease that degrades structural proteins and ultimately damages organelles (Zhang *et al.*, 2021). In mitochondrial dysfunction, several signs of damaged mitochondria in nodal cells were observed under TEM, including mitochondrial disruption, mitochondrial fission, numerous small dense mitochondria, and mitophagy. Under HG condition, mitochondrial ROS (mtROS) are highly produced, which increases mitochondrial permeability to induce mitochondrial swelling and cristae disruption, together with activated calpain 1, leading to impairment of ATP synthesis and ATPase pump function, especially the Na^+/K^+ ATPase pump, to maintain ion balance and osmotic equilibrium (Li *et al.*, 2009; Zhang *et al.*, 2021). These mechanisms can aggravate deteriorated organelles and osmotic stress in diabetic nodal cells. In accordance with mitochondrial damage in DM, mitochondrial fragmentation, characterized by a large population of small dense mitochondria together with mitophagy, was found. This appears to be an adaptive process to eliminate mitochondrial damage and maintain mitochondrial functions. Previous studies have been shown that mitochondrial fragmentation results from mitochondrial dynamic imbalance caused by changes in their regulatory proteins: increased level of fission protein and decreased level of fusion protein. High $[\text{Ca}^{2+}]_i$ level in DM activates dynamin related protein 1, a fission protein (Wu *et al.*, 2021). HG downregulates peroxisome proliferator-activated receptor alpha and in turn reduces the expression of the fusion protein mitofusin 2 (Hu *et al.*, 2019). As a result, mitochondrial dynamics are shifted toward fission rather than fusion to cause mitochondrial fragmentation, which is finally destroyed by mitophagy and/or autophagy.

Beyond osmotic stress and organelle deterioration, a clear cytoplasm in nodal cells observed under LM might be accompanied by the increased accumulation of glycogen granules in nodal cells. It has been suggested that glycogen accumulation in cardiomyocytes leads to cardiac hypertrophy and impedes intra- and intercellular electrical signal conduction, contributing to the development of arrhythmia. Despite the unclear mechanism underlying increased glycogen in the diabetic hearts, this phenomenon is speculated to be the compensatory response of critical tissue (heart, brain) for impaired glucose oxidation during metabolic disorder (Varma *et al.*, 2018). Interestingly, the suppressed activity of AMP-activated protein kinase (AMPK), which regulates cardiac energy metabolism, positively correlates with increased accumulation of cardiac glycogen in DM (Shearer *et al.*, 2011).

However, the STZ diabetic AVN also exhibited smaller nodal cells and shrunken nuclei together with the presence of macrophages. These signs can be linked to apoptosis. Correspondingly, evidence from an autopsy sample of type 2 diabetic patient with a 2° AV block has been demonstrated apoptosis in AVN tissue (Hostiuc *et al.*, 2013). As described before, DM provokes organelle damage and cell injury in several ways. Furthermore, the clearance of damaged organelles by autophagy and mitophagy is attenuated in both type 1 and type 2 DM, which is related to diabetic cardiomyopathy. This can be a cause of excessive cellular stress to trigger apoptosis. In autophagy, decreased phosphorylation of AMPK and increased phosphorylation of mammalian target of rapamycin inhibit autophagic activity in the diabetic heart. In mitophagy, downregulation of phosphatase and tensin homolog-induced putative kinase 1 and Parkin is associated with reduced mitophagy in diabetic hearts (Xu *et al.*, 2013; Kobayashi & Liang, 2015). Suppression of autophagy and mitophagy is responsible for the accumulation of damaged organelles and toxic misfolded proteins, which intensify nodal cell stress and trigger mitochondrial-dependent apoptotic pathway. Inordinate stress in cells activates proapoptotic protein Bak/Bax oligomerization to form mitochondrial outer membrane (MOM) pores. The opening of the MOM pore induces cytochrome C release into the cytoplasm to activate caspase 9 and 3 and finally undergo apoptotic changes (Chen *et al.*, 2020).

In addition, AVN nodal cells of diabetic rats showed the disrupted cell junction-induced nodal cell separation. This results form an increased interstitial collagen interposing the intercellular gap between neighboring nodal cells. In addition, the formation of an annular profile of gap junctions, termed connexosome, which represents the endocytic process or internalization of gap junctions, was observed. In STZ-induced-diabetic rats, electrical conductivity is suppressed, consistent with the reduction in connexin (Cx) 43 protein, the main gap junction protein. The elevated PKC- ϵ activity in DM mediates the hyperphosphorylation of Cx43, which undergoes endocytosis and proteolytic degradation, respectively (Lin *et al.*, 2006). These are suspected to be the cause of junctional disassembly and cell separation in nodal cells of AVN in DM.

An increase in collagen accumulation with a marked presence of myofibroblasts was detected in the AVN mass of diabetic rat. One main mechanism underlying extracellular matrix remodeling in the development of diabetic cardiomyopathy is activation of the transforming growth factor- β (TGF- β) signaling pathway. In DM, TGF β and its receptor (TGF β R) are overexpressed in the heart tissue (Du *et al.*, 2021). Binding of TGF β to TGF β R on cardiac

fibroblast membrane stimulates Smad-dependent and phosphoinositide 3-kinases/Akt/glycogen synthase kinase 3 β pathways to upregulate fibrotic genes; α -smooth muscle actin, Col I, and Col III (Shyu, 2017). Consequently, fibroblast to myofibroblast transdifferentiation and collagen deposits occurred in AVN in diabetic rats.

Morphological changes in AVN nerve innervation were found in diabetic rats, as evidenced by swollen axons, dilated axonal mitochondria with disrupted cristae and Schwann cell injury. Osmotic stress and increased ROS via HG-induced mechanisms, as mentioned above, are also potential causes of axon and Schwann cell injury in DM. Furthermore, Schwann cells interact with axons to provide metabolic support, ion regulation, and adaptive responses to injury. The injury of Schwann cells might decline glial-axon communication to compromise axon homeostasis, eventually causing axon loss, leading to neurodegeneration in DM (Bouçanova & Chrast, 2020).

CONCLUSION

Prolonged uncontrolled hyperglycemia in DM led to structural remodeling in the AVN of rats. Diabetic AVN presented signs of nodal cell injury and death, together with abnormal cell junctions and nodal cell separation. Moreover, the interstitial structures, including extracellular matrix and nerve, were also altered by DM. These events might be related to the impaired AP generation and conduction in the AVN in DM, which probably causes AV block.

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PLAENGRIT, K.; LANLUA, P.; NIYOMCHAN, A. & SRICHAROENVEJ, S. Diabetes no controlada con cambios estructurales subyacentes en el nódulo auriculoventricular. *Int. J. Morphol.*, 41(4):1027-1035, 2023.

RESUMEN: La miocardiopatía diabética, caracterizada por anomalías musculares cardíacas inducidas por diabetes mellitus (DM), es un fuerte inductor de alteración de la contracción cardíaca y arritmia. El bloqueo atrioventricular, un tipo grave de arritmia resultante de la interrupción de la conducción del impulso cardíaco a través del nodo atrioventricular (NAV), se produce con frecuencia entre los pacientes diabéticos. Sin embargo, los detalles

de los cambios estructurales en NAV en DM siguen estando pobremente explicados. Aquí, este estudio definió los efectos de la DM en la remodelación morfológica del NAV en ratas macho Sprague Dawley inducidas por inyección intraperitoneal de estreptozotocina (60 mg/kg de peso corporal). A las 24 semanas, los cambios patológicos en el NAV se evaluaron mediante microscopía óptica (MO) y microscopía electrónica de transmisión (MET). Bajo MO, el NAV en ratas diabéticas se convirtió en una masa menos compacta y exhibió la vacuolización intracelular. Las células nodales tenían tamaños más variados con ausencia o contracción de núcleos y citoplasma claro en comparación con el control. El contenido de colágeno aumentó significativamente en relación con la presencia de miofibroblastos. De acuerdo con MO, las imágenes MET de las células nodales diabéticas revelaron varios signos de daño celular, como cambios mitocondriales, deterioro de los orgánulos celulares, internalización de uniones comunicantes y separación celular. Además, también se encontraron cambios en la innervación del NAV, evidenciados por schwannocitos y axones dañados. Estos resultados indicaron alteraciones en componentes importantes en el NAV durante la condición diabética, lo que puede conducir al deterioro de la conducción eléctrica, causando funciones cardíacas anormales en estos pacientes.

PALABRAS CLAVE: Diabetes mellitus; Arritmia; Nodo atrioventricular.

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