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Genotoxicity of Streptozotocin versus High Fat Diet-Induced Hyperglycemia in Adult Albino Rat Myocardium

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Abstract. *Background*: Hyperglycemia has detrimental systemic and cardiac effects by increasing oxidative stress and advanced glycation end products (AGE). Prolonged hyperglycemia-induced DNA damage in various tissues. *Aim*: To compare the hyperglycemia-induced genotoxicity of STZ versus a high-fat diet (HFD) on rat myocardial tissue. *Methods*: Sixty-six adult male rats were allocated randomly into 3 groups of 22 rats each; Group I was maintained on a standard chow diet, Group II where rats were injected with STZ (40 mg/kg, intraperitoneally) for 5 consecutive days, and Group III where rats were fed HFD for 15 weeks. After reaching the hyperglycemic level (> 150 mg/dl), all rats were kept for 5 weeks on HFD to ensure the occurrence of DNA damage. Rats were euthanized and sacrificed at the end of the experiment. Blood samples were collected from the tail vein. The heart was excised carefully and processed for histological (Hematoxylin & eosin) and immunohistochemical staining (γH2AX). *Results*: The HFD group's serum levels were significantly higher for NO and MDA than the STZ group's serum levels, whereas GSH levels were lower. On histopathological examination, congested blood vessels, inflammatory cells, and spacing between cardiac muscle fibers were seen in Groups II and III. Regarding γH2AX immunostaining, the reaction was marked in Group III as compared with Group II. *Conclusion*: Hyperglycemia induced deteriorative changes in the myocardial tissue. HFD-induced hyperglycemia produced much genotoxic damage to the myocardium. This could be related to its dramatic oxidative damage.

Keywords: streptozotocin, high-fat diet, hyperglycemia, myocardium, genotoxicity

Introduction

Diabetes mellitus (DM) has a detrimental effect on the heart, resulting in diabetic cardiomyopathy and ischemic heart disease (Tsao *et al.*, 2022). Numerous processes are thought to be involved in diabetic cardiomyopathy (El Hayek *et al.*, 2021). One of these hypotheses holds that high blood sugar levels result in oxidative stress and advanced glycation end products (AGE), which have harmful effects on the body's organs and the heart (Pappachan *et al.*, 2013).

Streptozotocin (STZ) is a naturally occurring chemical derived from Streptomyces chromogens that are harmful to beta cells in the pancreas that produce insulin.. Treatment with (STZ) causes its preferential accumulation in the pancreatic cells, where it induces hyperglycemia through glucotoxicity caused by mitochondrial dysfunction (Ahmed *et al.*, 2017).

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A sedentary lifestyle is a documented fundamental risk factor for metabolic and cardiovascular illnesses (Booth *et al.*, 2012). Obesity, early hyperinsulinemia, impaired glucose homeostasis caused by insulin resistance, and late insufficient insulin production are all consequences of a high-fat diet (HFD). It also consists of approximately 58 percent of its calories from fat (Winzell & Ahrén, 2004). In research, HFD is widely used to make mice obese and develop type 2 diabetes, simulating how the environment might affect an animal's metabolism (Kuwabara *et al.*, 2017).

The prolonged hyperglycemic condition leads to pancreatic β-cell damage and induces insulin resistance. Hyperglycemia induces biochemical and DNA damage in the experimental rat's heart, liver, and kidney tissues (Aydın *et al.*, 2019). Oxidative stress can cause an array of deleterious consequences, including DNA strand breaks, protein cleavage, and membrane peroxidation, all of which can result in cancer (Collins & Harrington, 2002; Mittler, 2002). The most common type of DNA damage, known as oxidative DNA damage, also comprises DNA-protein bonding and DNA single- and double-strand breakage (Marnett, 2000; Bjelland & Seeberg, 2003; Cadet *et al.*, 2004). When DNA oxidation occurs frequently during replication, mutations may result. Wu *et al.* (2017) demonstrated that genotoxic effects occurred 5 weeks following hyperglycemia.

Our study handled the genotoxic effect of hyperglycemia on the myocardial tissue precisely. Also, it compares the hyperglycemia-induced genotoxicity of STZ versus genotoxicity of HFD.

Materials and Methods

Chemicals

Sigma Aldrich Chemicals Co. (St. Louis, MO, USA) supplied the streptozotocin (STZ). In citrate buffer STZ was dissolved (0.1 mol/L, pH 4.4). The feeding ingredients of high fat diet (HFD) "compromising of fat 46%, carbohydrates 24%, proteins 20.3%, fiber 5%, salt mixture 3.7%, and 1% vitamin mixture" were purchased from commercial sources. Rabbit polyclonal Anti-gamma Histone 2A.X antibody (γH2AX) was purchased from ABclonal Biotechnology Co. Ltd (Cat. No. A11540).

Calculation of Sample Size

Using the G*Power program (version 3.1.9.7), sample size was determined. Sixty sex albino rats (22 per group) from the three groups whose means are to be compared are used in a one-way ANOVA analysis. Using an F test with a 0.05 significance threshold, the whole sample of 66 subjects has 80% power. The effect size $\eta^2 = \sigma m^2 / (\sigma m^2 + \sigma^2)$, which is 0.40, represents the means extent of variation.

Ethical Approval

The "Guide for the Care and Use of Laboratory Animals" was followed for every experiment involving animals. These rules were set by the institute of Laboratory Animal Research and published by the National Research Council, USA, 2011. Under the legislation (VM.R.23.02.50), the Mansoura University- Animal Care and Use Committee (MU-ACUC) accepted the study protocol.

Experimental Animals

Sixty male albino rats (weighing 200–250 g) were used in this study. Rats were acclimated to standard laboratory conditions for a week before the experiment.

Experimental Design

Rats were erratically separated into 3 groups (each group = 8 rats): Group I (control group) remained on standard chow nutrition (51.93 g carbohydrate, 3.03 g lipid, 20.50 g protein, 4.17 g crude fiber/100 g diet, and 3.00 kcal/g energy) (Vatandoust *et al.*, 2018), Group II (STZ group) where rats received injections by STZ (40 mg/kg, intraperitoneally) for 5 consecutive days (Furman, 2015), and Group III (HFD group) where rats were fed HFD for 5 weeks (Wu *et al.*, 2017). Rats with fasting blood glucose (FBG) levels more than 150 mg/dl were included in the study (Furman, 2015) after having tail vein samples examined by the GlucoDrTM one-touch glucometer (Allmedicus, Korea). All rats were kept on HFD for 5 weeks to ensure that DNA damage happened after exceeding the hyperglycemic threshold (Wu *et al.*, 2017).

Blood Samples and Analyses

Rats were sacrificed after completion of the experiment. Blood samples were collected from tail veins. For 20 minutes, the samples were centrifuged at 4000 rpm and 4°C. In preparation for the subsequent measurement of oxidative stress indicators, sera were separated and kept at -20°C. The nitrate reductase assay was used to measure the amounts of NO in the serum. Thiobarbituric acid was used to measure MDA levels (Shi *et al.*, 2019) and Additionally evaluated was the non-enzymatic antioxidant biomarker GSH (Korany *et al.*, 2019).

Tissue Preparation

The heart was dissected just after the chest wall was breached to collect blood. Isolated cardiac tissue was fixed in 4% paraformaldehyde for 24 hours.

Histopathological Study

Transverse sections were prepared, processed, and paraffin-embedded after being fixed in formalin 10% (Sigma-Aldrich, St. Louis, USA). Hematoxylin-Eosin (H&E) stain was applied to sections (5 m thick) for regular histopathological analysis (Lee *et al.*, 2020).

Immunohistochemical Detection of Myocardial Genotoxicity

Paraffin sections (5μm thick) were deparaffinized in xylene and rehydrated with adding 0.03% hydrogen peroxide (H2O2). Following a 20-minute boil in 0.01 M citrate buffer (pH 6), samples were subjected to 10% horse serum/PBS for 20 minutes at room temperature before any extra serum was removed from the experiment. The primary antibody was incubated on the sections using polyclonal antibody for (H2AX) (1:50). After using 3,30-diaminobenzidine (DAB) (Abcam cat. N 64264) and horseradish peroxidase -conjugated goat antirabbit polyclonal antibody as a secondary antibody, tissue slice examination was carried out using light microscopy (Elhessy *et al.*, 2023).

γH2AX Image Analysis

In each group, five sections were checked. Area percentage of Polyclonal antibody for (γH2AX) positive reactions in the myocardium was measured. An immune-positive reaction was examined using the image analysis programme Image-j (version 1.48). Green, Brown, and Blue were three distinct colours that were produced using the H-DAB vector and the colour deconvolution plugin. Estimating area fraction was used to calibrate the DAB photographs, which are brown in hue (Hassan *et al.*, 2022).

Statistical Analysis

The data was collected and analyzed using the Statistical Package for the Social Sciences (SPSS) program, version 26 for Windows 10 (Chicago, IL, USA). The variables, which were presented as mean standard deviation (SD), were analyzed using one-way analysis of variance (one-way ANOVA), and Tukey post-hoc analysis was performed to compare the two groups. P-values below 0.05 were regarded as significant. Charts were created using the Windows 11 version of Microsoft Excel.

Results

Results of the Biochemical Analysis of Serum Oxidative Stress Markers

Serum levels of NO, MDA, and GSH were assessed to compare the oxidative stress between the two model groups compared to each other and compared to control. Our study demonstrated that NO and MDA levels significantly increased in STZ and HFD groups compared to control groups. But the HFD group showed a dramatic increase compared to STZ group. On the other hand, there was a significant decline in GSH in both model groups in contrast with control, but the HFD showed a significant decline compared to STZ group (Figure 1).

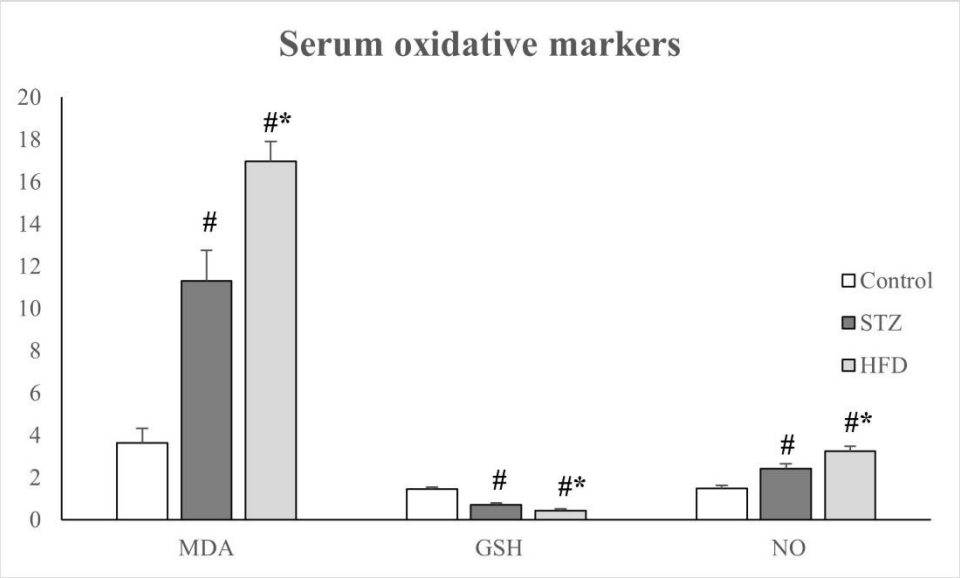

Figure 1: Evaluation of serum oxidative stress markers in studied groups

Note: Comparison between groups was performed via One way ANOVA followed by post hoc Tukey's test, #: Compared to control group (p value (≤0.001), *: Compared to STZ group (p value (≤0.001). Abbreviations: MDA, Malondialdehyde; NO, Nitric oxide; GSH, reduced glutathione; STZ, streptozotocin; HFD, high-fat diet.

Histopathological Assessment of the Cardiac Muscle Architecture

The myocardium in the control group exhibited a normal architecture. The central, oval, and vesicular nuclei of the acidophilic cytoplasm were visible in the heart muscle fibers. Meanwhile, the STZ-treated group demonstrated some congested blood vessels and relative wide spaces among the cardiac muscle fibers. In addition, HFD-treated group showed markedly congested blood vessels with perivascular inflammatory cellular infiltrate. There was wider spacing among the cardiac muscle fibers (Figure 2).

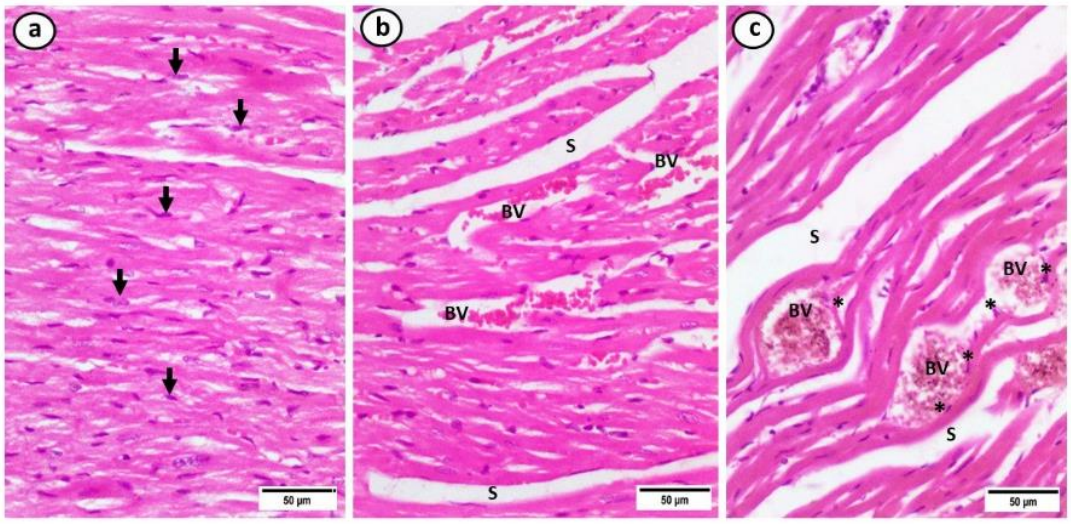

Figure 2: Hematoxylin and eosin-stained cardiac muscle tissue among studied groups

Note: Photomicrographs of myocardial sections showing**; (a)** Control group with the normal architecture of the myocardium. The central, oval, and vesicular nuclei (arrows) of the acidophilic cytoplasm were visible in the heart muscle fibers. **(b)** STZ-treated group showing some congested blood vessels (BV) and relatively wide spaces among the cardiac muscle fibers (S). **(c)** HFD-treated group showing markedly congested blood vessels (BV) with perivascular inflammatory cellular infiltrate (stars). There were wider spacing in-between the cardiac muscle fibers (S). **(H & E, x 400)**

Immunohistochemical Assessment of γH2AX (Recombinant Anti-gamma Histone 2A.X Antibody) among Different Experimental Groups *in the Myocardium*

In our study, photomicrograph of control group showed faint γH2A immunoreactivity in fewer myocardial nuclei. On the other hand, STZ-treated group showed mild positive γH2AX immunoreactivity in some myocardial nuclei. HFD-treated group strong positive γH2AX immunoreactivity in most of myocardial nuclei (Figure 3).

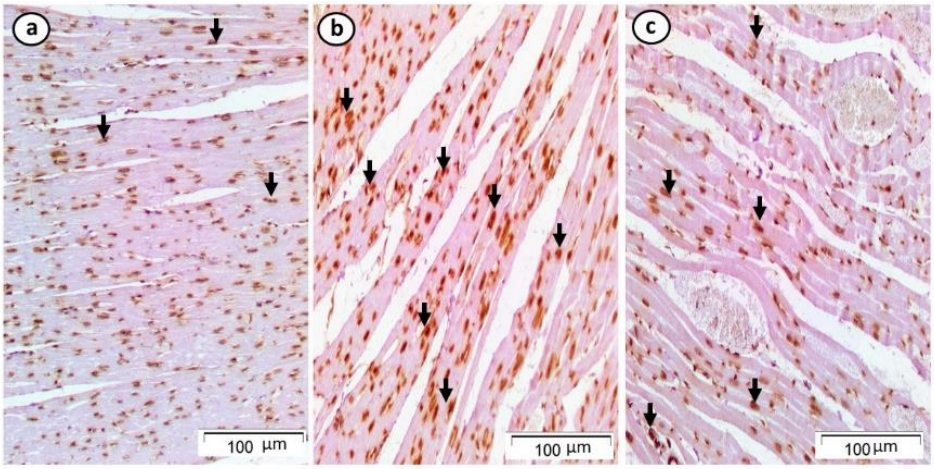

Figure 3: γH2AX Immunohistochemical stained myocardial sections showing; (a) Control group with faint γH2AX immunoreactivity in fewer myocardial nuclei (arrows). **(b)** STZ-treated group showing mild positive γH2AX immunoreactivity in some of the myocardial nuclei (arrows). **(c)** HFD-treated group showing strong positive γH2AX immunoreactivity in most myocardial nuclei (arrows). **(γH2AX Immunohistochemical, x 200)**

Morphometric Results

Both STZ and HFD groups showed significant elevation in γH2AX expression compared to control group. Noted that, HFD group demonstrated significant rise in of γH2AX expression compared to STZ group (Figure 4).

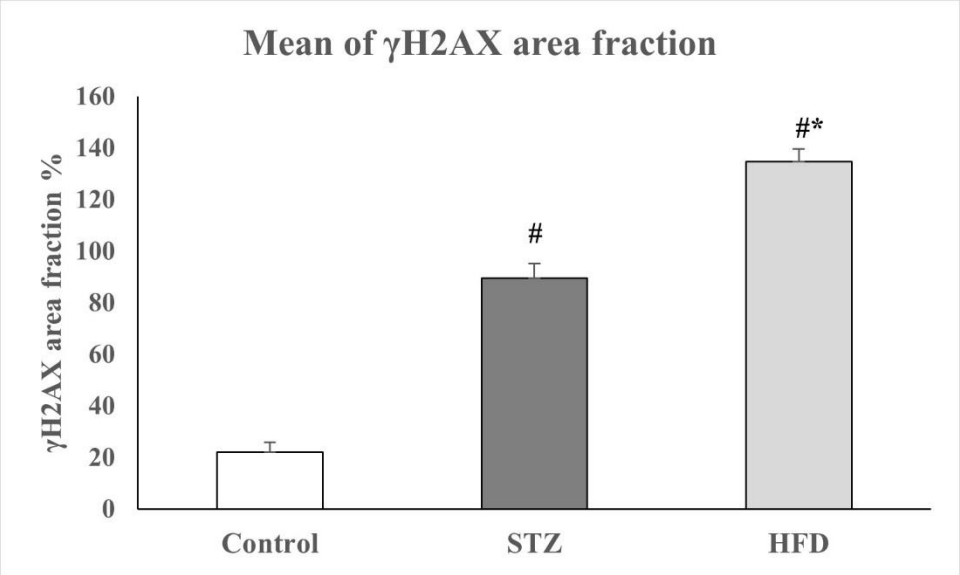

Figure 4: The Morphometric results of γH2AX reaction in different experimental groups

Note: Comparison between groups was performed via One way ANOVA followed by post hoc Tukey's test, #: Compared to control group (p value (≤0.001), *: Compared to STZ group (p value (≤0.001).

Discussion

One of the main diabetic consequences and the main cause of death in those who have diabetes is diabetic cardiomyopathy (DCM) (Carpentier, 2018). Even in the absence of additional risk factors, long-term hyperglycemia may deteriorate myocardial function (Boudina & Abel, 2007). By activating various oxidative pathways, hyperglycemia promotes the generation of reactive oxygen species (ROS) (Costantino *et al.*, 2018). According to (Battiprolu *et al.*, 2012), ROS cause myocardial inflammation, mitochondrial derangement, apoptosis, fibrosis, and contractile failure through activating transcriptional programs.

A significant rise in oxidative stress is observed in animal models of obesity set by high-fat diet (HFD) even in the early stages of the condition (Luc *et al.*, 2019; Akkoc *et al.*, 2021). Inflammation, decreased insulin secretion, action, and a development of cardiovascular disease follow this. Determining the genotoxic impact of hyperglycemia on the cardiac tissue was the main objective of the current study. Additionally, it contrasted the genotoxicity of STZ driven by hyperglycemia to that of HFD.

Both STZ and HFD groups revealed a significantly MDA and NO level rise and a significantly GSH level decline in compared to the control group when it was assessed for oxidative stress (p = 0.001). The STZ results concurred with those of Tripathi and Chandra (2009), Farshid *et al.* (2016), Fouda *et al.* (2019). According to Amin and Nagy (2009), Mattapally and Banerjee (2011), He *et al.* (2012), HFD causes oxidative stress in obese rats. These results for the HFD group verified these findings. As a result of increased nicotinamide and flux of flavin adenine dinucleotides into the respiratory chain of the mitochondria caused by hyperglycemia and insulin resistance, the mitochondrial inner membrane becomes hyperpolarized, complex III's electron transport is inhibited, and too much ROS is produced

(Teshima *et al.*, 2014). Diet-induced obesity has been linked to increased cardiomyocyte nicotinamide adenine dinucleotide phosphate oxidase activity (Jia *et al.*, 2016).

According to Chang *et al.* (2015), the highly unstable aldehyde MDA is a sign of oxidative stress. In cardiac myocytes, hyperglycemia may raise the level of ROS. Too much ROS will lead to oxidative stress, lipid peroxidation, and the generation of harmful MDA (Xiang et al., 2020). According to Suryawanshi *et al.* (2006), increased protein glycation in diabetes mellitus may be the cause of increased lipid peroxidation. It is obvious that lipid peroxide and glucose concentration are related, and it is possible that this relationship contributes to increased lipid peroxidation seen in diabetes mellitus.

Barrientos *et al.* (2021) confirmed that elevated MDA suggests that chronic consuming too much food can temporarily increase glucose and circulating and intracellular lipids due to glycolipotoxicity, which contributes to insulin resistance and β−cell damage, and that there is a positive correlation between weight gain and elevated pancreatic MDA. Elevated MDA also signals the initial stage of cell damage.

Through enhanced expression of the eNOS and iNOS gene and protein levels, elevated glucose levels may promote NO production (Zhang *et al.*, 2014). Depending on the NO concentration, increased levels of NO in vivo could have either a positive or a negative effect. Rapid superoxide radical (O2) and nitric oxide (NO) interaction results in the production of the strong oxidant radical peroxynitrite. By promoting the metabolism of arachidonic acid, lipid peroxidation, and prostanoid synthesis, this may lead to poor endothelial function (Ishii *et al.*, 2001). According to Ataie *et al.* (2021), glucose-stimulated insulin secretion increased the Ca++ concentration inside the cells, which naturally boosted the islet NO synthase (NOS) activity. According to Fujimoto et al. (2005), feeding rats a high-fructose diet caused the examined iNOS expression to increase.

Reduced glutathione (GSH), meanwhile, is well known for guarding the biological system against the harmful consequences of lipid peroxidation. In addition to serving as a cofactor for numerous enzymes and a direct free radical scavenger, GSH also forms conjugates in endo- and xenobiotic processes. The idea that advanced glycation end products, free radical production rates, and polyol pathways are all regulated by persistent hyperglycemia is supported by a number of research. Aldose reductase (AR) activation in diabetic rats' cardiac tissue results in a relative depletion of NADPH. Additionally, the detoxification of lipid peroxidation byproducts including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the form of GSH aldehyde adducts has been linked to the action of AR. All these may lead to depleted GSH levels in heart tissue (Tripathi & Chandra, 2009).

Additionally, hyperglycemia is linked to oxidative stress because it triggers an inflammatory response and oxidative damage in the target tissue via ROS- and NOX1-mediated glycoxidation (Vlassara & Striker, 2011; Yang *et al.*, 2011)

The myocardium specimens from the STZ group's histological analysis showed specific blood vessels that had become congested and with large gaps between heart muscle fibres. According to El-shaer and Nofal (2019); Wang *et al.* (2020), these findings were consistent. Oxidative stress can be used to explain myocardial affection since it plays a significant part in the pathophysiology of DCM by damaging myocardial cells and molecules, which results in defects in heart structure and function (Cai & Kang, 2001).

Blood vessels in the HFD group were noticeably congested and displayed inflammatory cellular infiltration around them. In line with Li et al. (2017), the heart muscle fibers were spaced farther apart (Yan *et al.*, 2009). The accumulation of Reactive oxygen species in heart tissue during insulin resistance, which occurs before diabetes develops, has been linked to cardiac dysfunction through lipid peroxidation, extracellular matrix remodeling, mitochondrial damage, DNA damage, and changes in coupling proteins (Mellor *et al.*, 2010; Tsutsui *et al.*, 2011). Cardiac slices from the STZ group in the current investigation had

mildly positive H2AX staining in some cardiac nuclei. The findings of Savi *et al.* (2016); Marino *et al.* (2022); Rostoka *et al.* (2023)were all in agreement with this result. According to Marino et al. (2022), the hyperglycemic condition increases the amount of DNA damage brought on by oxidative stress, which results in altered production of indicators including 3-nitrotyrosine (3-NT), 8-hydroxy-20-deoxyguanosine (8-OHdG), and γ H2AX (a DNA damage marker). Additionally, these indicators have been linked to DM heart cellular senescence processes.

Additionally, Faria and Persaud (2017) found that changed glucose and fatty acid metabolism in the heart led to nitric oxide rise, which interacted with increased free radicals to generate a hazardous molecule—peroxynitrite—which contributes for damage to major biological molecules, including DNA. The integrity of the DNA molecule in the heart is consequently directly impacted by oxidative and nitrosative stress in diabetes, and DNA breaks occur (Ivanović-Matić *et al.*, 2014).

According to Azzarà *et al.* (2017), who reported increased γ H2AX-positive cells noticed in obesity rats due to the constitutive hyperglycemic condition, which was expressed in the gut, esophagus, and pancreas, γ H2AX stained section of the HFD group showed strong positive immunoreactivity in most myocardial nuclei compared to control. The increased γ H2AX phosphorylation in fatty animals is indicative of obesity, which can result in double strand breaks (DSB), which can lead to organ damage.

Conclusion

The HFD group was shown to have higher levels of oxidative stress indicators, myocardial histological alterations, and a lower γ H2AX area percentage than the STZ group. This means that the hyperglycemia caused by HFD was more genotoxic to the myocardium than the hyperglycemia caused by STZ. More investigation would be beneficial to find out about additional pathways behind the genotoxic consequences of hyperglycemia. Additionally, genotoxicity as well as hyperglycemia itself must be the focus of antihyperglycemic medication use.

Authors Contributions

Hend M. Hassan and Heba M. El-Hessy designed the study. Hend M. Hassan examined the myocardial tissue specimens, interpreted the histological & immunohistochemical results. Heba M. El-Hessy performed and interpreted the morphometric results. Nehal H.M. Abdel-Halim and Dina Salem established the study model. Ola Ali Habotta was concerned with validation and methodology.

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