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Heat Stress Induces Oxidative Stress and Predisposes Rats to Gestational Diabetes Mellitus

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Abstract

Gestational diabetes mellitus (GDM) is a form of hyperglycemia due to carbohydrate intolerance that begins during pregnancy. This may be due to insulin resistance or impairment of insulin secretion during the pregnancy. Several causes of GDM have been identified which

include oxidative stress (OS), however the association of heat stress and GDM development during pregnancy is limited. Therefore, this study aimed at examining the association between heat stress and GDM in rats. Pregnant and non-pregnant Wistar rats were maintained at 41 - 42°C for 21 days. On day 1, 8, 15 and 21 of the experiment, animals were humanely sacrificed. Blood samples for glucose, insulin, malondialdehyde (MDA) and glutathione peroxidase (GPx) analyses were collected from the heart. Pancreatic tissues were fixed in neutral buffered formalin, and processed for histopathological studies. The findings demonstrated that, in pregnant rats, heat stress induced a significant increase in glucose linked with a drop in insulin levels than non-pregnant rats (P <0 .05). Also heat treatment was accompanied by an increase in MDA and a drop in GPx levels. Histological examinations of the pancreas revealed damaged β-cells on day 15 and reduction in the number of β-cells by day 21 of the experiment in the pregnant rats. These results suggest that heat stress raises the levels of OS in pregnant rats than non-pregnant rats and increases the chance of GDM as it is associated with β-cell defects in the pancreas.

Keywords: pregnancy, heat stress, glucose, insulin, malondialdehyde, glutathione peroxidase

1. Introduction

Gestational diabetes mellitus (GDM) is a form of hyperglycemia in mammals brought on by a carbohydrate intolerance that begins during pregnancy (Nanobashvili *et al.*, 2018). Insulin resistance (Boloker *et al.*, 2002; Genuth *et al.*, 2015) and impaired insulin secretion (Punthakee *et al.*, 2018) during pregnancy are among the causes of the onset of GDM. If not effectively managed GDM is associated with a short and long-term health risks to the mother, developing fetus and the offspring (Wainstock & Yoles, 2019).

Studies have shown that oxidative stress (OS) is one of the factors contributing to insulin resistance and the development of GDM (AbdulAziz *et al.*, 2016; Li *et al.*, 2016; Feng *et al.*, 2020). This can be due to overproduction of free radicals (reactive oxygen species (ROS)) and impairment of the antioxidant system during pregnancy (Murthy *et al.*, 2018). Under OS there is an occurrence of a chain reaction called lipid peroxidation which results into formation of several active compounds that can lead to cellular damage. The degree of lipid peroxidation can be estimated by the amount of malondialdehyde (MDA) in tissues, hence a biomarker for oxidative stress (Cui *et al.*, 2018). Likewise, there are antioxidant enzyme systems in the body that scavenge the ROS produced. These include superoxide dismutase (SOD), catalases (CAT) and glutathione peroxidase (GPx). The defensive system of these enzymes can fail if the production of ROS in the body increases beyond normal, and the condition leads to OS (Singh *et al.*, 2014). Overproduction of ROS and the resultant OS can cause impairment of insulin signaling and insulin sensitivity which end up with hyperglycemia, leading to diabetes mellitus (Seshiah *et al.*, 2011; Hurrle & Hsu, 2017). Furthermore, OS was observed to increase as pregnancy progresses (Lappas *et al.*, 2011; Murthy *et al.*, 2018; Feng *et al.*, 2020).

Other factors associated with the increase in OS include diet, obesity (Vega *et al.*, 2016), radiation exposure, smoking, alcoholism and environmental temperature (Ngoula *et al.*, 2020). Environmental temperature, particularly heat stress, is linked with pregnancy complications (Samuels *et al.*, 2022). This is among the reasons for an increase in the prevalence of GDM

during summer season (Preston *et al.*, 2020). However, there is limited information on the association between OS due to heat stress with glucose and insulin levels in different stages of pregnancy and GDM development. Hence, the current study is aimed at exploring the role of OS due to heat stress during pregnancy in GDM development using the Wistar rat model.

2. Material and Methods

2.1 Study Area

The experiments were carried out at the Small Animal Research Unity (SARU) in the College of Veterinary Medicine and Biomedical Sciences (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

2.2 Ethical Clearance

The Animal Research Ethical Committee (RPGS/R/ETHICS) of SUA approved the use of animals in this experiment. Thus, appropriate protocols for the handling and use of experimental animals were used.

2.3 Experimental Animals

This study used female Wistar rats, which were procured from SARU. They were housed in cages in a clean room at 25 ± 3 °C, 35 - 60% relative humidity and a 12/12 hours light-dark cycle. During this period, the animals were maintained on standard pellet food and *ad libitum* drinking water. Rats were observed for estrous cycles as per AbdulAziz *et al*., (2016), and mating was done to those following four (4) day cycles at a ratio of 1:2 (male: female). The presence of a vaginal plug was indicative of positive mating and was regarded as pregnant and noted as day 0 of gestation (GD 0) (Mbepera *et al.*, 2023). Those without plugs were considered not-mated and thus non-pregnant animals.

2.4 Experimental Setup and Animal Treatment

Sixty-four (64) pregnant and non-pregnant female Wistar rats were used in this experiment (**Table 1**). For the induction of heat stress (HST), 32 pregnant and non-pregnant animals were subjected to 41 - 42°C throughout the experiment (21 days). The other 32 pregnant and non-pregnant animals were maintained at room temperature (25 ± 3°C) throughout the experiment. In the course of gestation, 4 animals from each group were humanely sacrificed by administration of ketamine (50 mg/kg) and xylazine (5 mg/kg) on day 1, 8, 15 and 21 (**Table 1**) and samples were collected.

Table 1. Experimental setup

Groups	Treatment	Number of sacrificed animals				**Total**
		Day 1	Day 8	Day 15	Day 21	
1	Pregnant (P+)	4	4	4	4	**16**
2	Non-pregnant (P-)	4	4	4	4	**16**
3	Heat stress pregnant (HSTP+)	4	4	4	4	**16**
4	Heat stress non-pregnant (HSTP-)	4	4	4	4	**16**
Total		**16**	**16**	**16**	**16**	**64**

2.5 Sample Collection

Whole blood was collected by cardiac puncture from the sacrificed animals into plain and heparinized vacutainer tubes for the analysis of glucose, insulin, MDA and GPx. The collected blood samples were centrifuged for 12 minutes using a centrifuge (MPW M-Diagnostic, model; M-universal, Poland). Serum and plasma were transferred into Eppendorf tubes and kept at -20°C until the time for analysis. Pancreas was trimmed out for histopathological processing and examination.

2.6 Analysis of Biochemical Parameters

2.6.1 Glucose

Trinder's methods Endpoint kit (Erba Mannheim GmbH, India) was used for glucose analysis. The absorbance of standard and samples were read at 505 nm by using Unico Spectrophotometer (1100RS, Cole Parmer®, USA), and glucose concentration was calculated as:

$$Glucose\ conc\ \left(\frac{mg}{dl}\right) = \frac{Absorbance\ of\ test}{Absorbance\ of\ standard} \times Conc\ of\ standard\ \left(\frac{100mg}{dl}\right)$$

2.6.2 Insulin

Rat insulin ELISA Kit (Colorimetric) NBP3-00515 (Novus, USA) was used to determine insulin levels. The optical density (OD) of the samples was measured spectrophotometrically at 450 nm, using Microplate Spectrophotometer (EPOCH™, Biotec®, USA). The duplicate readings for each standard and sample were averaged and subtracted by the average of zero standard optical density. The standard curve was constructed with the OD values on the y-axis and standard concentrations on the x-axis, then used to compute the concentrations of insulin in the samples.

2.6.3 Malondialdehyde (MDA)

The Universal Malondialdehyde kit, NBP2-78753 (Novus, USA) was used for the determination of the Malondialdehyde levels. Determination of enzyme-substrate reaction was done by adding stop solution and colour change measured spectrophotometrically at 450 nm to obtain the OD using a Microplate Spectrophotometer (EPOCH ™, Biotec®, USA). The duplicate readings for each standard and sample were calculated. The standard curve was constructed with the OD value on the y-axis and standard concentrations on the x-axis, then used to calculate concentrations of MDA in the samples.

2.6.4 Glutathione Peroxidase (GPx)

Glutathione peroxidase was assayed by using Rat GPX1 (Glutathione Peroxidase 1) Kit, NBP2-68016 (Novus, USA). The enzyme-substrate reaction was determined by the addition of stop solution and colour change measured spectrophotometrically at 450 nm to obtain the OD by using Microplate Spectrophotometer (EPOCH ™, Biotec®, USA). The duplicate readings for each standard and sample were calculated. The standard curve was constructed with OD values on the y-axis and standard concentrations on the x-axis. The OD value was proportional to the concentration of Rat GPX1, therefore the concentrations of Rat GPX1 in the samples were calculated by comparing the OD of the samples to the standard curve.

2.7 Histopathological Analysis of Pancreas

Tissue samples from the pancreas were fixed in 10% neutral phosphate-buffered formalin overnight and processed routinely. Tissue blocks were obtained after embedding in the paraffin wax. Blocks were sectioned to obtain 4 µm sections, which were stained with Hematoxylin and Eosin and mounted by using dibutylphthalate polystyrene xylene (DPX). The examination of the sections was done by using a light microscope (Olympus Corporation, U-DO3, S/N 9M11951, Tokyo, Japan) and photographs were taken by camera mounted to the microscope.

2.8 Data Handling and Analysis

The descriptive data analysis for means and standard error of the mean (SEM) were performed using SPSS version 25. Data normality was evaluated using the Kolmogorov-Smirnov test. Mann-Whitney test was employed as a non-parametric test, while Two-Way ANOVA and Tukey's test were used as parametric tests. The differences between groups were considered significant at $p \leq 0.05$.

3. Results

3.1 Glucose, Insulin, MDA and GPx Levels

The levels of glucose, insulin, MDA and GPx in pregnant (P+) and non-pregnant (P-) Wistar rats exposed to HST are shown in **Figure 1**.

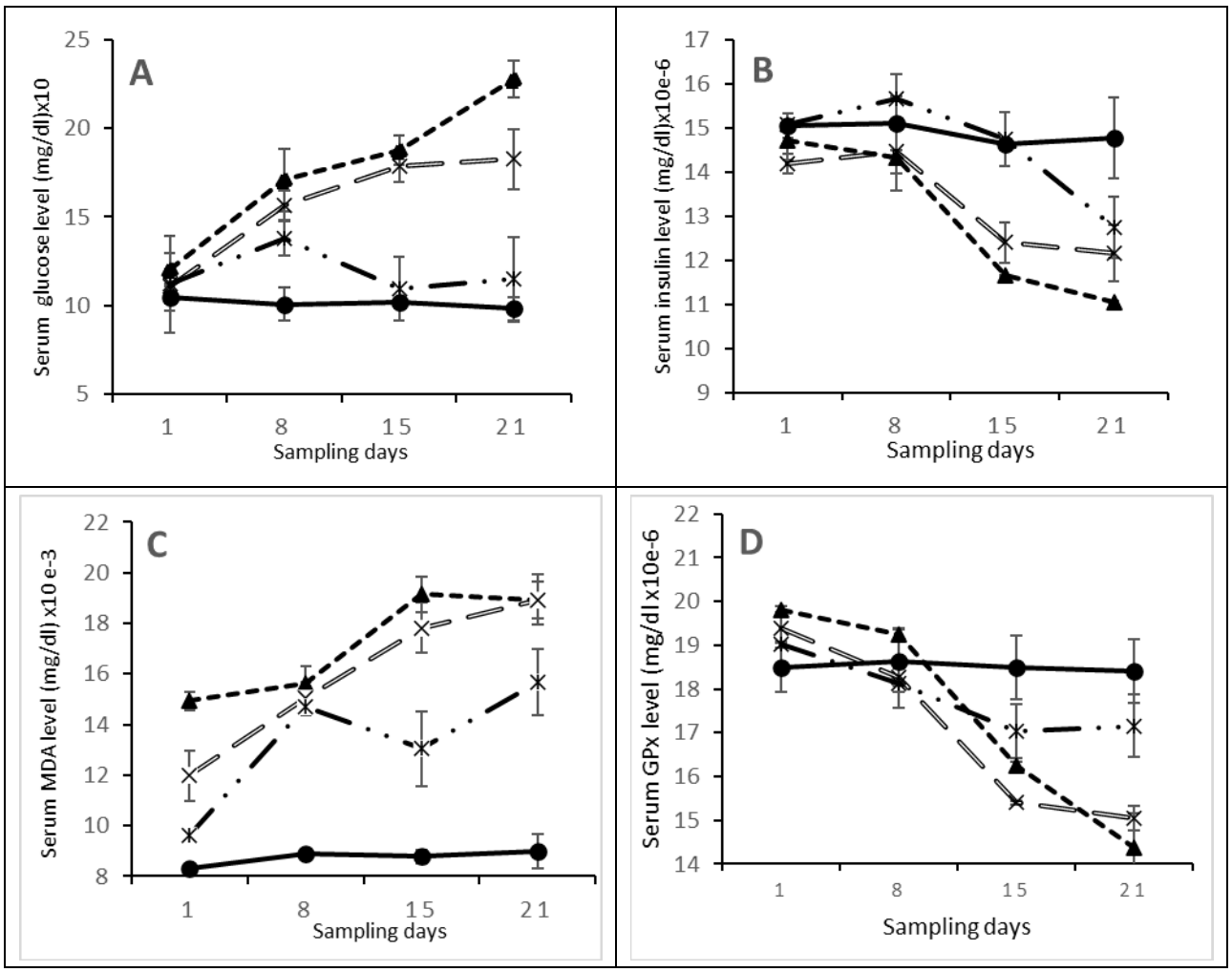

Figure 1. Serum levels of glucose, insulin, MDA, and GPx from pregnant and non-pregnant Wistar rats exposed to HST. Values were Mean ±SEM. Pregnant rats ( ), non-pregnant rats ( ), pregnant rats treated with HST ( ) and non-pregnant rats treated with HST ( )

Figure 1A demonstrates that throughout the experiment, HST caused high glucose levels in both pregnant and non-pregnant rats. Day 1 glucose levels in HSTP+ treated animals were 1.2±0.095 $\times 10^2$ mg/dl equal to 1.09 times their control (P+). The levels increased significantly ($p<0.05$) to about 1.75±0.174 $\times 10^2$ mg/dl on day 8 equal to 1.3 folds over the control. The levels peaked up to 2.28±0.105 $\times 10^2$ mg/dl equal to 2.0 folds over the control on day 21 of the experiment. In HSTP- animals, the levels of glucose on day 1 was 1.1±0.095 $\times 10^2$ mg/dl equal to their control value. The levels increased significantly ($p<0.05$) on day 8 to 1.56±0.084 $\times 10^2$ mg/dl equal to 1.6 times their control values. On day 15 glucose levels increased to about 1.8±0.09 $\times 10^2$ mg/dl equal to 1.8 folds over the control, then remained the same on day 21 of the experiment. HST induced a significantly higher level of glucose ($p<0.05$) in HSTP+ than HSTP- on day 21 of the experiment.

The levels of insulin in HSTP+ and HSTP- animals are presented in **Figure 1B**. Up to day 8 of the experiment the levels of insulin were 1.45±0.05 $\times 10^{-5}$ mg/dl equal to 1.06 folds under their controls. The levels decreased significantly ($p<0.05$) on day 15 to 1.17±0.0013 $\times 10^{-5}$ mg/dl in

HSTP+ and 1.24±0.045 $\times 10^{-5}$ mg/dl in HSTP-, compared to 1.47±0.06 $\times 10^{-5}$ mg/dl value of their control (P+), then remained the same on day 21 of the experiment. Significant ($p<0.05$) difference in insulin levels between HSTP+ and HSTP- was observed on day 15 and 21 of the experiment.

Levels of MDA increased in HSTP+ and HSTP- animals throughout the experiment (**Figure 1C**). In HSTP+ animals, the day 1 level of MDA was 1.5±0.036 $\times 10^{-2}$ mg/dl, equivalent to 1.6 times their control value, which remained the same up to day 8. The levels then increased ($p<0.05$) on day 15 to 1.91±0.071 $\times 10^{-2}$ mg/dl, equal to 1.5 folds over their control, then remained the same throughout the experiment. In HSTP- treated animals, the levels of MDA were 1.2±0.099 $\times 10^{-2}$ mg/dl on day 1, equal to 1.4 folds over their controls. Levels then increased slightly to 1.5±0.067 $\times 10^{-2}$ mg/dl on day 8, equal to 1.7 folds of the control. A further significant increase was observed on day 15 to 1.8±0.099 $\times 10^{-2}$ mg/dl equivalent to 2.0 times the control, which remained the same to day 21 of the experiment. The levels of MDA were significantly higher in P+ than P- groups throughout the experiment; however, the levels were significantly higher on day 1 and 15 in HSTP+ than in HSTP-.

GPx levels decreased differentially with time in all animal groups (**Figure 1D**). Both pregnant and non-pregnant animals exposed to HST, had GPx levels of 1.9±0.05 $\times 10^{-5}$ mg/dl, similar to their controls. In HSTP+ treated animals GPx levels were the same on day 8, then decreased significantly ($p<0.05$) to 1.62±0.009 $\times 10^{-5}$ mg/dl on day 15 equal to 0.95 folds less than the control and to 1.44±0.078 $\times 10^{-5}$ mg/dl equal to 0.8 fold less than their control value on day 21. In HSTP- animals, there was a slight decrease in GPx levels to 1.83±0.02 $\times 10^{-5}$ mg/dl on day 8 equal to 0.95 folds under their control. Then the levels decreased significantly ($p<0.05$) on day 15 to 1.5±0.005 $\times 10^{-5}$ mg/dl equal to 0.83 folds under their control, then remained constant on day 21 of the experiment. The levels of GPx were significantly higher in P- than P+ on day 15 and 21 of the experiment; however, the levels were significantly higher on day 1 and 15 in HSTP- than in HSTP+.

3.2 Histopathology of the Pancreas

The histological structures of the pancreatic islets and the β-cells from rats subjected to HST are represented in **Figure 2** (P+) and **Figure 3** (P-). On day 1, the pancreatic islets and β-cells in HSTP+ and HSTP- animals were similar to the controls (**Figure 2** and **3**). However, β-cells of the pancreatic tissue section from HSTP+ and HSTP- animals on day 15 were undergoing pyknosis. The section from HSTP+ revealed more giant cells and pyknotic cells on day 15 (**Figure 2**) while vacuolation and necrosis were more evident on day 21 leading to patches of cellular loss. However, HSTP- showed pyknotic cells on day 15 and vacuolation on day 21 (**Figure 3**).

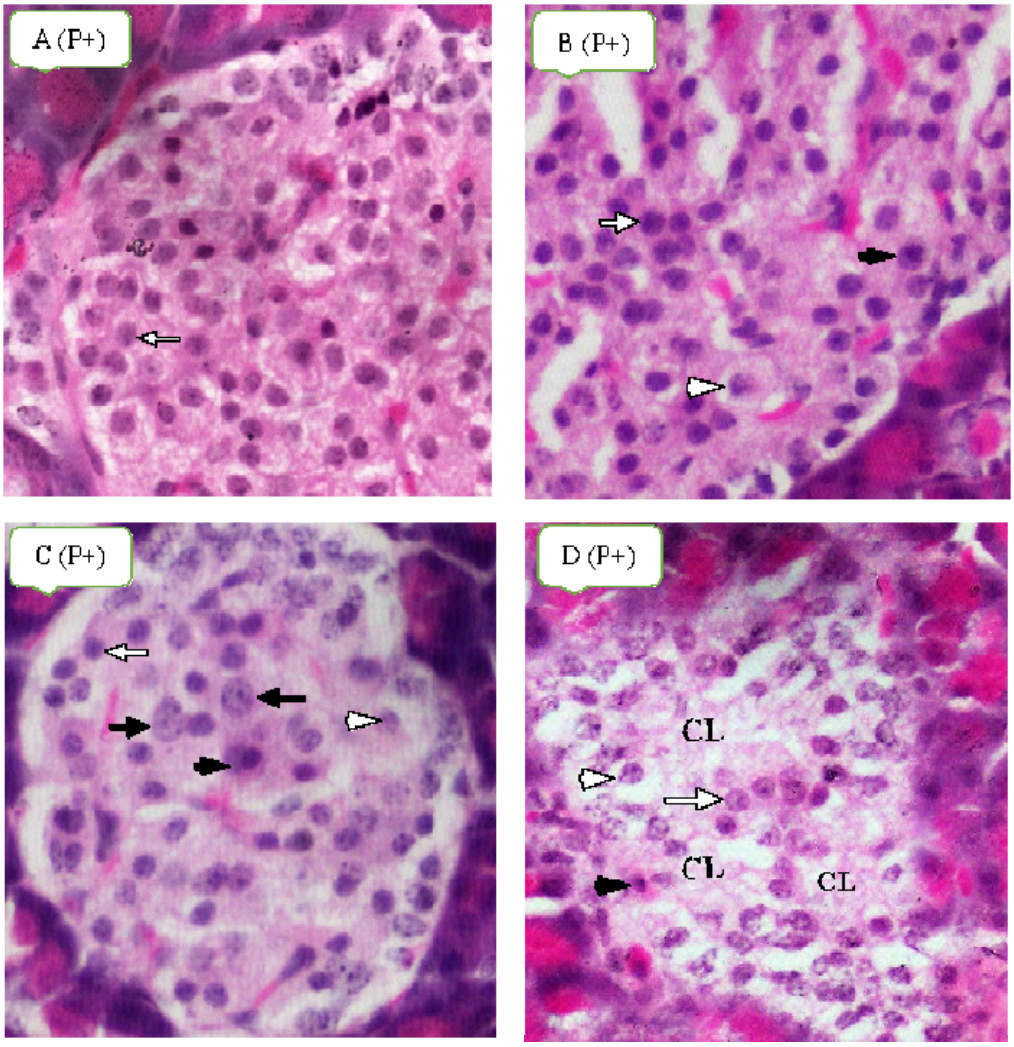

Figure 2. Histopathology of the section of the pancreas (H & E, × 400) from pregnant Wistar rats of (A) control, (B) HST - day 1, (C) HST - day 15, (D) HST - day 21. (⇨) normal cells, (➡) giant cells (➤) cells that undergo pyknosis (▷) cells that undergo vacuolation, and (CL) patches of cellular loss

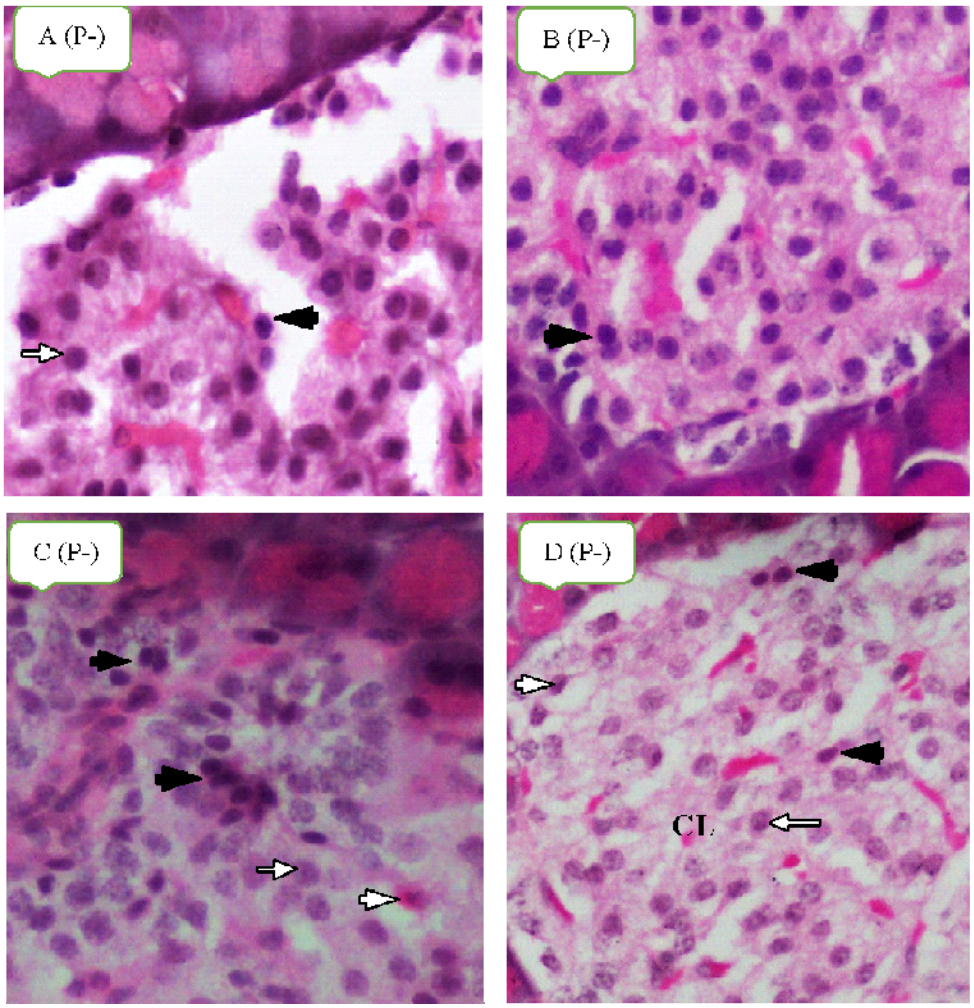

Figure 3. Histopathology of the section of the pancreas (H &E × 400) from non-pregnant Wistar rats of (A) control (B) HST - day 1, (C) HST- day 15, (D) HST- day 21. (⇨) normal cells (➨) cells that undergo pyknosis (⇦) cells that undergo vacuolation, and (CL) patches of cellular loss

4. Discussion

In HST P+ and P- groups, there was an increase in the levels of glucose and a decrease in insulin with gestation. Findings similar to these were reported in Europe by Vasileiou *et al.*, (2018) who observed that an increase in temperature above 25°C is associated with an increase in plasma glucose values in humans. Also, according to Blauw *et al.*, (2017), the greater outdoor temperatures were associated with an increase in the prevalence of glucose intolerance globally in humans and the rate of diabetes in the USA. Similarly, Pace *et al.*, (2021) found that women in Australia, Canada, Sweden and the United Kingdom had greater rates of GDM in summer than in winter seasons. The current study suggests that heat stress prompts β-cell destruction. Clearly, we observed a trend of islet cell destruction during gestation due to heat treatment (**Figure 2**). This destruction went hand in hand with insulin levels reduction (**Figure 1B**) and hyperglycemia (**Figure 1A**). However, this observation contrasts slightly with that of Retnakaran *et al.*, (2018) who linked a high prevalence of GDM with β-cells dysfunction

during months of high ambient temperature. On the other hand, Valdés *et al.*, (2019) reported that a rise in the surrounding temperature was related to insulin resistance. Also, according to Hurrle & Hsu, (2017), the increase in ambient temperature may result in OS which has a strong association with insulin resistance and GDM. Moreover, Yaribeygi *et al.*, (2020) observed that OS increased apoptosis of pancreatic cells including β-cells, decreased β-cell neogenesis and altered metabolic pathway leading to β-cell dysfunction. The lesions in endocrine pancreases (patches of cellular loss) which were more extensive in HSTP+ than in HSTP- in the current study could have been the reason for the differing levels of insulin and glucose between the two groups. The suggested mechanism behind the damaged islet cells in the current study is the increase in free radicals due to heat stress which promoted cellular necrosis. An increased level of MDA (**Figure 1C**) was an indication of high levels of reactive oxygen species leading to a reduction of GPx levels (**Figure 1D**). Related results were reported by Ilievska *et al.*, (2016) after exposing young and adult male rats to 41 - 42°C heat for 60 minutes leading to increased levels of free radical species as reflected by elevated MDA levels.

5. Conclusion

The current study has clearly indicated that HST during pregnancy could promote the development of GDM in Wistar rats through increased levels of OS. The OS was associated with defects in the pancreatic β-cells, which could have an effect on the normal way by which the body produces and uses insulin during pregnancy. Further studies are required to investigate the involvement of other factors such as cytokines which are produced during heat stress and their contribution in GDM development.

Declaration of Conflict of Interest

Authors declare no conflicting interest regarding the publication of this paper.

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