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Research Article

Long-Term Hyperglycaemia Impairs Hormonal Balance and Induces Oxidative Damage in Ovaries of Streptozotocin-Induced Diabetic Wistar Rat

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Summary: Reproductive dysfunction following insulin deficiency in Diabetes Mellitus has been well reported among diabetic patients. However, the mechanism through which Diabetes alters reproductive function remains oblivion. While most studies have focused on diabetes mellitus in male subjects, there have been cases on altered reproductive functions in females. These present study aims to investigate the effect of long term hyperglycemia on diabetic rats' ovary. Female Wistar rats were assigned into control and diabetic group, each consisting of five animals. The later was induced with STZ (50mg/Kg intraperitoneal injection) and the animals were sacrificed after 14 weeks. The blood glucose, body and organ weight, serum hormone level along with oxidative stress parameters of the ovary and uterus were determined. Histology of the ovary and expression levels of CD79 in the ovary was also assessed. The weight of the diabetic rats after the experiment was significantly lower ($p<0.05$) than the control. The level of Follicle Stimulating Hormone, Luteinizing hormone and estrogen was significantly lower in the diabetic group. The antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione-s-transferase (GST) were significantly lower in the diabetic ovary and uterus while the Malondialdehyde (MDA) concentration significantly increased compared to the control group. Histological observation of the ovary showed signs of chronic inflammation and immunohistochemistry for CD79 showed positive expression in the diabetic ovary. Our research findings suggest that Diabetes mellitus alters ovarian health by altering hormonal balance and stimulating oxidative damage.

Keywords: *Diabetes mellitus, Oxidative stress, Hormonal imbalance, ovarian dysfunction, CD79, Streptozotocin*

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INTRODUCTION

Diabetes mellitus play a critical role in different pathological conditions in humans, including reproductive dysfunction (Chatterjee *et al*., 2013). Females with Diabetes mellitus encounter impaired reproductive functions, although the data are not well established as compared to male (Steger & Rabe, 1997). Diabetic Mellitus is associated with decreased fertility and reproductive losses (Nandi & Poretsky, 2013). Popoola *et al*. reported a biochemical, hormonal and histological change in the prostate of male diabetic rats after three months and six months (Popoola *et al*., 2017).

Several perturbations occur in female diabetic patients ranging from reduced ovulation, low embryo development, abnormal oocyte maturation and altered metabolism (Qiang Wang and Kelle H. Moley, 2016). Of significant note is the increased susceptibility to polycystic ovarian syndrome (PCOS) and menstrual irregularities associated with type 1 diabetes patients (Codner *et al*., 2012). Since the ovaries play a significant role in these functions, an extensive study would provide insight into the pathologies of infertility in female diabetic patients.

Steger and Rabe (1997) reported that diabetes mellitus disrupt endocrine function. According to Tesone *et al*. (1983), decreased hCG receptors may be responsible for ovarian dysfunction in diabetic animals. Deepika and Zaher observed a link between increased advanced glycation end product in diabetic women and increased susceptibility to PCOS (Garg & Merhi, 2015). Zhao *et al*. (2010) reported that Diabetes increased testicular apoptotic cell death via the mitochondria pathway and demonstrated that oxidative stress is the leading cause of testicular detrimental effects. These suggest that reactive oxygen species and hormonal imbalance may play a

significant role in reproductive organ failure in female Wistar rats.

B-lymphocyte has been reported to play a critical role in type 1 diabetes as depletion of B-cells delays diabetes progression in diabetic patients and NOD mice (Hinman *et al*., 2014). While most of these damage has been attributed to CD20 lymphocyte, not much is known about the role of CD79 in Diabetes progression. Hence, this study is aimed at providing insight into the role of oxidative stress and hormonal imbalance and to evaluate the possible role of CD79-B-cell receptor-mediated apoptosis and proliferation in the ovary of diabetic rats.

MATERIALS AND METHODS

Chemicals and Reagents: Streptozotocin was purchased from Santa Cruz, USA. Glutathione, Hydrogen peroxide and 5,5'-dithios-bis-2-nitrobenzoic acid (DNTB) were purchased from Sigma Chemical Company, Saint Louis, USA. Thiobarbituric acid (TBA) and Trichloroacetic acid (TCA) were purchased from British Drug House (BDH), Chemical Limited, Poole, UK. Immunohistochemistry was performed using the Novocastra Kit. Other chemicals and reagents used were of pure quality and analytical grade.

Experimental Animals: Female Wistar rats weighing 130-150g were purchased from the Department of Veterinary Physiology, University of Ibadan. The rats were kept in ventilated cages and housed at room temperature 25±2^{0}C with 12h dark/light cycles. Animals were fed standard rat pellet and were acclimatized for two weeks before the experimental treatment. Animals were handled according to standard ethical procedures stipulated by the Animal Care Unit Research Ethics Committee (ACUREC), University of Ibadan.

Experimental Design: Female Wistar rats were assigned into two groups.

Group1: Control rats fed with standard rat pellet only

Group 2: Diabetic rats that were administered 50mg/Kg body weight intraperitoneal STZ injection.

Rats with blood glucose above 250mg/dl after 72h post-STZ injection were termed diabetic. Animals that remained in this hyperglycemic state for a prolonged duration of 14 weeks were used for the experimental analysis.

The animals in each group were eventually sacrificed, the blood samples were collected in plain bottles, and the uterus and ovary were exercised. The organs weighed, and the percentage relative organ weight was calculated as a ratio of the organ weight to the bodyweight of the animal expressed in percentage. The blood glucose of the animals was determined using a glucometer and standard test strip.

The tissues collected were weighed, and 50% fraction of it was homogenized in four volumes of phosphate buffer and centrifuged at 10,000rpm in a cold centrifuge at 4^{0}C for estimation of oxidative stress parameter. The other tissue section was fixed in 10% formalin for histopathology and immunohistochemistry studies.

Hormonal Assay: FSH (Follicle Stimulating Hormone) and LH (Luteinizing hormone) levels were determined using a solid based enzyme-linked immunosorbent assay as described by Uotila *et al*., (1981), estrogen (EST) and Prolactin (PRL) concentrations were determined by the ELISA method described by Norbert *et al*., (1991).

Oxidative stress Estimation: Protein determination: Protein levels were determined according to the method of Randall and Lewis (1951) using bovine serum albumin as standard.

- The activity of Superoxide dismutase (SOD) was determined according to the method described by Mistra & Fridovich (1972).
- Catalase (CAT) activity was determined using the method of Claiborne Claiborne (1985)
- Glutathione-s-transferase (GST) activity was determined using the method of Habig *et al.* (1974).
- Lipid peroxidation was determined based on Malondialdehyde (MDA) concentration using the method of Buege and Aust (1978).

Histology: Ovarian samples were fixed in Bouin's solution, dehydrated in 95% ethanol and cleared in xylene before embedding in paraffin. 5 µm section were cut, stained with hematoxylin and eosin dye and examined under a light microscope (×400) by a histopathologist who was ignorant of the treatment groups.

Immuno-Histochemical Assay: Fresh ovary sample from control and diabetic rats was fixed in formalin and embedded in paraffin and cut to a thickness of 7µm section. The sections were deparaffinized, rehydrated and immuno-staining for expression of CD79 was performed using mouse primary antibody (Novocastra, New Castle Upon Tyne, England) (Bhargava *et al*., 2007) dilution of 1:100.

Negative and tonsil positive control slides were stained alongside the experimental slides. Results were assigned qualitative values: negative(-), weak (+), moderate (++), strong (+++) based on expression levels of CD79a protein (Fedchenko & Reifenrath, 2014).

Statistical Analysis
Quantitative data were compared using a two-tailed student t-test for data analysis using Graph Pad Prism 5 (Graph Pad Inc. San Diego, USA). The significant difference was set at $p< 0.05$ and results reported as mean ± SD.

RESULTS

Effect of long-term diabetes on Bodyweight and organ weight of reproductive organs: Long term exposure hyperglycemia in female Wistar rats resulted in a statistically significant ($p<0.05$) decrease in body weight compared to the control rat, which showed an increase in body weight. Also observed was a statistically significant decrease in the weight of ovary and uterus compared to the control. The relative percentage weight of the uterus was also statistically different as compared to the control rats ($p<0.05$).

Table 1:
Effect of long-term diabetes on body weight and reproductive organ weight in female Wistar rats

	Measure	Control	Diabetic
Body Weight	Initial	150.0 ±2.59	150.0 ±4.86
	Final	204.14 ±19.65	127.58 ±24.65*
Ovary Weight	Absolute Weight	0.14 ±0.045	0.061 ±0.007*
	% Relative Weight	0.07 ±0.02	0.05 ±0.01
Uterus Weight	Absolute Weight	0.40 ±0.09	0.116 ±0.01*
	% Relative Weight	0.19 ±0.04	0.095 ±0.03*

Values are expressed as mean ± standard deviation (n=5).
** Statistically different compared to control (p<0.05).*

Effect of Long-term diabetes on blood glucose level in female Wistar rats: The final blood glucose of diabetic rats showed a significant increase ($p<0.05$) from the initial blood glucose before STZ induction and from the final blood glucose of the control rats (Figure 1).

Effect of long term diabetes on endocrine levels in female Wistar rats: As shown in Figure 2, the FSH level, LH level and estrogen level of the diabetic rats showed a statistically significant decrease in the plasma of the diabetic rats compared to the control. The level of prolactin was, however, not statistically different compared to control.

Effect of Long term diabetes on Oxidative stress parameters in reproductive organs: The activities of the antioxidant enzyme catalase, superoxide dismutase and Glutathione-s-transferase were significantly lower in the ovary and uterus of the diabetic rats as compared to the control. A resulting marked significant increase in the concentration of Malondialdehyde (MDA) in the ovary and uterus of the diabetic rats as compared to the control was also observed (Table 2).

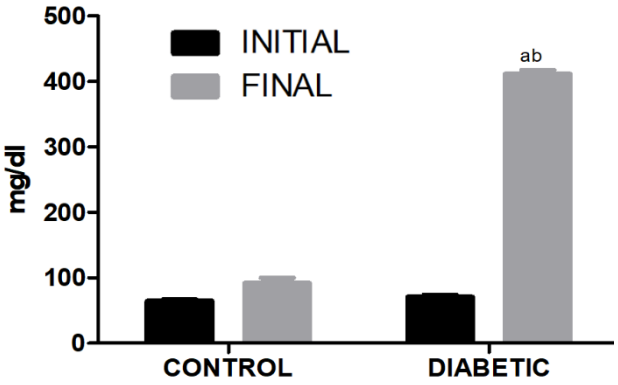

Figure 1:
Effect of long term diabetes on blood glucose level
Values are expressed as mean ± SD.
[a] statistically significant compared to control. [b] Statistically different from initial blood glucose ($P<0.05$).

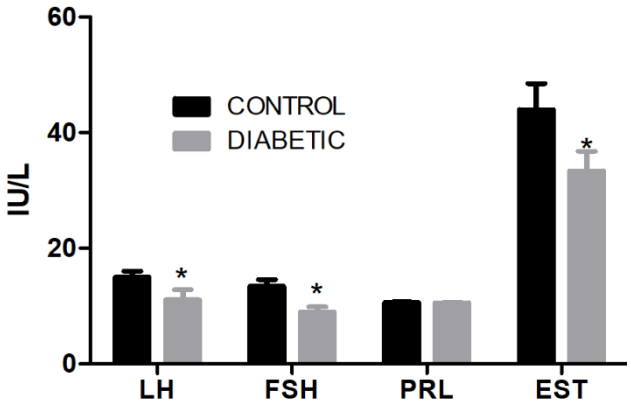

Figure 2:
Effect of long-term diabetes on the level of female reproductive hormones in Wistar rats. Values are expressed as mean ± standard deviation (n=5). * Statistically different compared to control ($p<0.05$).

Table 2:
Effect of long-term diabetes on oxidative stress parameters in ovary and uterus

Measure	Tissue	Control	Diabetic
SOD	Ovary	11±1.95	3.7±0.29*
	Uterus	15.3±0.47	9.46±0.98*
GST	Ovary	8.4±0.58	1.1±0.16*
	Uterus	10.66±0.59	6.54±0.32*
CAT	Ovary	11.62±0.28	3.62±0.40*
	Uterus	5.08±0.24	3.3±0.27*
MDA	Ovary	8.6±0.39	2.2±0.2*
	Uterus	4.36±0.24	0.72±0.13*

Values are expressed as mean ± standard deviation (n=5).
** Statistically different compared to control (p<0.05).*

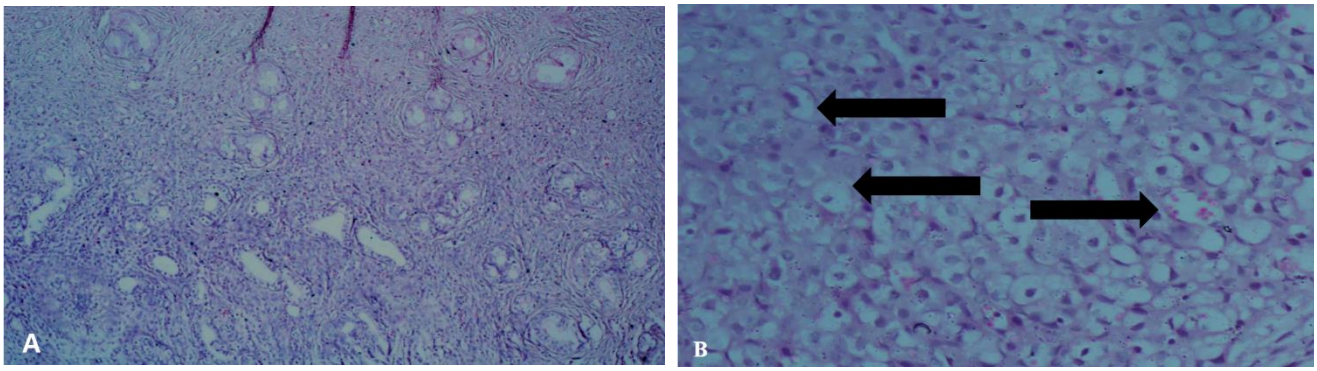

Plate 1:
Histology of the ovary in control and diabetic rats (×400)
A- Control rat ovary showing normal physiology; B-Diabetic rat ovary showing regions of chronic inflammation

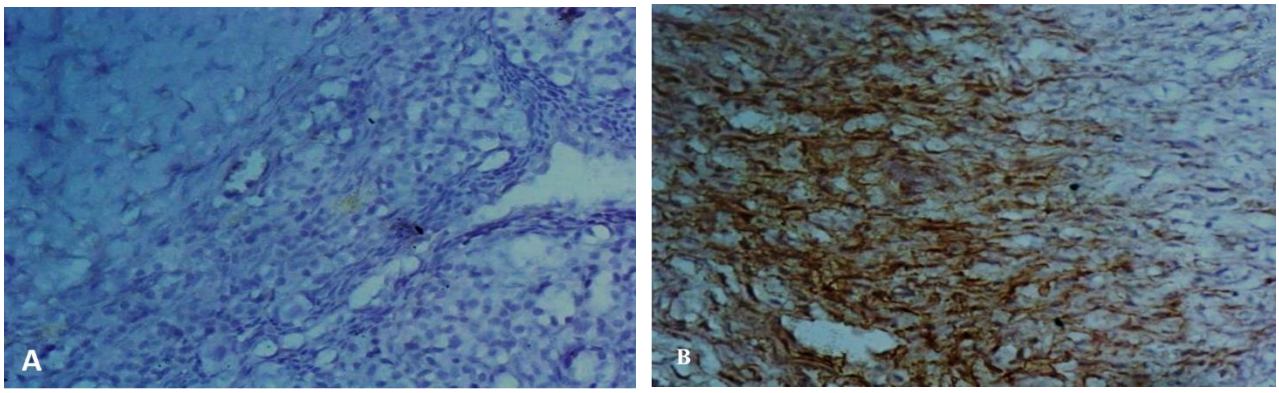

Plate 2:
Expression of CD79a in control and diabetic rats ovary(×400).
A. Control rats showing negative (-) CD79 expression.
B. Diabetic rats were showing moderate positive (++) CD79 expression, as shown by the dark bands. The pattern of staining was cytoplasmic and granular.

Effect of long-term diabetes on histology of the ovary: As shown in Plate 1, the ovaries of the control rat showed normal morphology while the histopathology of the diabetic ovaries showed signs of chronic inflammation of the ovarian cells.

Effect of Long term diabetes on the expression of CD79 in the ovary: The result shows that CD 79a is expressed transiently in the ovary of the diabetic rats and unexpressed in the control rats (Plate 2).

DISCUSSION

Female Patient with Diabetes mellitus has frequently been reported to have various reproductive irregularities resulting in infertility. This dysfunction has been linked to chronic hyperglycemia since the administration of insulin restores the physiological condition of the reproductive organs (Tesone *et al*., 1983). Previous studies have focused mainly on reproductive damage in male diabetic rats (Steger & Rabe, 1997). Owing to the link between prolonged hyperglycemia and reproductive dysfunction, female Wistar rats with prolonged chronic hyperglycemia were studied to evaluate the morphology and physiology of the ovary.

A single-dose administration of 50mg/Kg body weight STZ led to a significant increase in blood glucose of rats in the diabetic group. These state of chronic hyperglycemia remained till the end of 14 weeks before the animals were sacrificed. This is because a single high dose administration of STZ induced diabetes mellitus by destroying pancreatic beta cells (Szkudelski, 2001).

The diabetic rats showed a significant decrease in weight which is similar in human subjects with complications of Diabetes. This is similar to what was observed by Dekel *et al*., who reportedly observed about 40% decrease in weight 14 days post-STZ treatment in mice(MC Deeds, JM Anderson, AS Armstrong, DA Gastineau, HJ Hiddinga, A Jahangir, NL Eberhardt, 2014),(Dekel *et al*., 2009). This is due to massive tissue wasting resulting from hyperglycemia which stems from diabetic ketoacidosis (Umpierrez, 2018).

Decreased ovary and uterus weight can be attributed to a decrease in body mass in diabetic rats. However, the significant decrease observed in the relative weight of uterus indicates an underlying pathological condition. Also, Diabetes has been

reported to decrease the thickness of the myometrium (Tatewaki *et al*., 1989).

Female Diabetes is associated with amenorrhea and disorders with gonadotropin release (Steger & Rabe, 1997). Follicle-stimulating hormone is responsible for ovarian follicle growth, a decreased level in the diabetic rats compared to control rat can affect fertility in diabetic rats. Luteinizing hormone triggers ovulation, and decreased levels in diabetic rats was also observed. Also observed was a significant decrease in estrogen level compared to control. Studies have shown that hypogonadism is associated with chronic hyperglycemia (Komaki *et al*., 2005). In our study, ovarian atrophy (decrease in ovary mass) arises from prolonged hyperglycemia, causing a consequent decline in sex hormones levels.

The level of GST, CAT, SOD, which is antioxidant enzymes were significantly decreased in the diabetic rats and increased MDA concentration in the diabetic rats indicates oxidative stress. This agrees with previous studies conducted in the past, which indicates that oxidative stress contributed to gonadal degeneration in STZ-induced Diabetes in animal models (Shrilatha & Muralidhara, 2007). Prolonged hyperglycemia in the female rat increases free radical concentration resulting in damage in the ovary. The increased reactive oxygen specie concentration may result in accumulation of advanced glycation end-product in granulosa and theca cells resulting in polycystic ovarian syndrome (Diamanti-Kandarakis *et al*., 2007).

The chronic inflammation observed in the cytoplasm of ovarian cells from the histology is a hallmark of cellular necrosis (Allen *et al*., 2005). Hyperglycemia can cause accumulation of advanced glycation end-product resulting in activation of pro-inflammatory marked in the ovarian tissue, which can lead to Polycystic ovarian syndrome (Garg & Merhi, 2015). Female ovarian follicles also undergo apoptosis which is a necessary step for the regulation of follicular pool in the ovary (Vaskivuo & Tapanainen, 2003). However, apoptosis in ovary must be limited to prevent follicular imbalance which can result in infertility. Hyperglycemia has been reported to activate caspases thus increasing the rate of apoptosis (Wu *et al*., 2017), but to the best of our knowledge, the role of CD79 mediated apoptosis in the ovary has not been reported.

Asmita and Vrinda reported an immune-mediated degeneration of ovarian follicular cells via CD45 (Choudhury & Khole, 2015). In our research, we observed a moderate expression of CD79a in diabetic ovary. CD79 is a signalling component B-cell receptor (BCR) commonly used as a marker for leukaemia but can also be expressed in non-b-cell malignancies (Bhargava *et al*., 2007). It is composed of two major component CD79a and CD79b; both of the components have been reported to function synergistically by dimerization stimulating the B-cell receptors (Chu & Arber, 2001). BCR cross-linking has been reported to induce apoptosis or drive cell division in the presence of T-cells (Chu & Arber, 2001). Lower expression of estrogen levels and low tissue mass of ovary of diabetic rats suggest that CD79 expression may play a role of apoptosis in diabetic rat cells rather than cell division. However, the inflammation observed in the ovary gives credence to a possibility of CD79 mediating proliferation in ovary, thereby causing PCOS.

Results from this work showed that prolonged hyperglycemia in STZ induced diabetic in female Wistar rats alters the level of sex hormones via oxidative stress in the ovary which causes damage to ovarian follicular cells which may ultimately lead to infertility. Further studies should be carried out to validate the apoptosis mechanism in the ovary of long term diabetic rats, the expression of CD79 and its role in PCOS and offers new hope for therapeutic targets.

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