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Pregnancy and Fetal Outcomes Following Metformin Use in Diabetic Rats

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ABSTRACT: The female reproductive system is responsible for pregnancy and supports development of the fetus. The incidence of diabetes mellitus (DM) is increasing rapidly worldwide (Wild *et al.,* 2000). Metformin is an oral antidiabetic drug and it seems to have properties that could be beneficial in managing reproductive complications of DM. The present study determined the effect of metformin on pregnancy and fetal outcomes in Alloxan-induced diabetic female Sprague-Dawley rats. Eighty (80) pregnant rats were divided into four groups (20 rats per group). Implantation and pregnancy were first ascertained, and fetal outcome was observed and recorded. The measurement of plasma levels of βhCG, estradiol, progesterone, corticosterone and C-reactive peptide (CRP); were carried out on days 7, 14, 19 and at term. Results were analyzed using ANOVA and Newman Keuls post hoc test with statistical significance taken at $p<0.05$. Results showed a significant ($p<0.05$) increase in plasma level of progesterone and estradiol with a significant reduction in CRP levels in the treated groups. The number of fetuses and average litter size was also significantly reduced ($p<0.05$) in the untreated diabetic rats. No physical abnormalities were observed in litters from all the experimental groups. In conclusion, metformin administration during pregnancy confers maternal and fetal protective effects.

Keywords: Pregnancy, Fetal outcomes, Diabetes mellitus, Hormones, Alloxan

Introduction

Diabetes mellitus (DM) is a condition in which the body does not produce enough, or does not properly respond to insulin, a hormone produced in the pancreas. Insulin enables cells to absorb glucose for ready conversion to energy. In diabetes, the body either fails to properly respond to endogenously produced insulin, does not make enough insulin or both; this leads to glucose accumulation in the blood, resulting in various complications (Rother *et al*., 2007). In 2000, according to the World Health Organization (WHO), at least 171 million people worldwide (2.8% of the population) suffered from DM. Its incidence is increasing rapidly, and it is estimated that by 2030, this number will almost double (Wild *et al*., 2000). DM occurs throughout the world, but it is more common in the more developed countries. The greatest increase is, however expected to occur in Asia and in Africa, where most patients will probably be found by 2030. DM has been associated with reproductive impairment in both men and women.

Inadequate management of Type I DM may increase the risk of spontaneous abortion. Also Type 1 diabetic women with initial glycohemoglobin concentrations in pregnancy above 12% or median first trimester pre-prandial

glucose concentrations above 120mg/dl have an increased risk of abortions and malformations (David *et al*., 2013). Maternal DM, whether pre-gestational (Type I or II) or gestational in origin or appearance, may create an intrauterine environment of hyperglycemia that promotes fetal insulin secretion and growth beyond the already rapid increase in fetal weight expected during the last trimester of pregnancy (Tzilos*et al*., 2013). This leads to the higher incidence of macrosomia commonly observed for pregnancies complicated by DM (Winkoop *et al.,* 2015).

Metformin is the first-line drug for the treatment of Type II DM, particularly in overweight and obese peopleand those with normal kidney function (International Diabetes Federation 2005, National Institute for Health and Clinical Excellence; 2008). It is the most popular anti-diabetic drug (Drug Tropics 2007, Top Generic Drugs by Units 2006) and it is one of the only two oral anti-diabetics in the World Health Organization Model list of Essential Medicines (World Model List of Essential Medicines 2007). Metformin has recently become recognized as potentially beneficial for women with infertility and polycystic ovarian syndrome.

Metformin is usually employed for management of hyperglycemia in Type II DM. The present study seeks to explore the hypoglycemic and also, non-hypoglycemic properties, and the effect(s) of these on the complications usually associated with reproductive functions in Type I diabetic female rats.

Materials and Methods

Animals: Adult female rats (120-130g) of the Sprague Dawley strain were obtained from the animal house of The College of Medicine University of Lagos. They were kept under a 12hr light: 12hr dark cycle and were allowed free access to food and water throughout the experimental period. Animal identification was done in the Department of Cell Biology and Genetics, University of Lagos. All guidelines with the use and care of laboratory animals were strictly adhered to in accordance with the Institutional Animal Care Use Committee (IACUC).

Animal Grouping and Experiment: Rats were divided into four groups (n =20) of (I) control (distilled water); (II) diabetic/non-treated (distilled water); (III) metformin/non-diabetic (100 mg/kg daily); and (IV) metformin/diabetic (100 mg/kg daily). Implantation and pregnancy were ascertained, and fetal outcome was observed and recorded. The measurement of plasma levels of βhCG, estradiol, progesterone, corticosterone and C-reactive peptide (CRP); were carried out on days 7, 14, 19 and at term.

Drug Preparation and Administration: Metformin powder (obtained from Sigma-Aldrich Inc, USA) was suspended in distilled water to yield a concentration of 25mg/ml. The dose selected was 100 mg/kg daily (Choi *et al*., 2006). Administration lasted throughout the period of pregnancy (21-23 days).

Induction of Diabetes Mellitus: Diabetes was induced in the morning after fasting the animals overnight by a single intraperitoneal injection of 100 mg/kg of 10% w/v alloxan monohydrate (Sigma-Aldrich, Inc, USA) dissolved in distilled water. After 72 hours, blood glucose level of the dosed rats was checked and those with blood glucose level more than 200 mg/dl were considered, selected and used for the study.

Mating and Induction of Pregnancy: Cycling diabetic and non-diabetic rats in proestrus were isolated and mated on the evening of proestrus. The presence of a sperm plug in the rat vagina (as well as the presence of sperm cells in the vagina smear of the rat) the following morning, confirmed mating. This day was then recorded and taken as day 1 of pregnancy.

Determination of Implantation Sites: 1mg/ml of Evans Blue was injected via rat tail veins and allowed to circulate for about 15 minutes. Animals were then euthanized and then dissected; the implantation sites in the fallopian tubes observed and counted on day 7 of pregnancy (Dey, 2003).

Studies on Pregnancy and Fetal Outcomes: Fifteen pregnant rats from each group were sacrificed on days 7, 14 & 19 of pregnancy, while the remaining rats were allowed to deliver. The number of implantation sites was counted on day 7. The placental weight, number of fetal resorption and fetal weight were taken and recorded on days 14 and 19. Gestation length, litter size and birth weight were counted and recorded in the rats allowed to deliver. The head length and crown-rump length were measured and the live/birth index calculated. Also, the presence of cleft palate, number of limbs and digits were observed and counted by physical examination.

Determination of Hormone Levels: Enzyme linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Co., Ltd) was used for the determination of insulin, estradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), C-reactive peptide, corticotrophin releasing hormone (CRH) and βhcg. ELISA analysis was conducted during the phases of estrous cycle and in pregnant rats on days 7, 14 and 19 of pregnancy.

Statistical Analysis: Values represent mean ± standard error of mean (SEM). The values were analyzed by one-way ANOVA followed by Students' Newman-Keuls Post-hoc test using the Graphpad Prism 6 software. Differences were considered statistically significant at $p<0.05$.

Results

Pregnancy and Fetal Outcomes: 100% of the non-diabetic rats had a positive pregnancy diagnosis. 40% of the rats assigned to the diabetic groups had a positive pregnancy diagnosis. 1.3% of the pregnant rats in group IV died. During the entire period of pregnancy, control and non-diabetic rats had normal glycemic values (80-100mg/dl) and glycemia remained between 200-400mg/dl in the diabetic rats. Percent weight gain during pregnancy in the groups was; I (43.66±1.32), II (30.43±0.98), III (43.48±0.86) andIV (31.3±0.65). When compared to control, significant reductions ($p<0.05$) were recorded in groups II & IV with only an insignificant increase ($p>0.05$) in group III. No significant ($p>0.05$) change was observed in the gestation length when the experimental groups were compared to control. A significant ($p<0.05$) reduction was recorded in the number of fetuses in group II (4.8±0.30)when compared to control (8.5±0.39), with an insignificant ($p>0.05$) decrease in the remaining experimental groups. This was also expressed in the average litter size at birth with similar reductions in group II (4.9±0.21) when compared to control (8.1±0.11). The highest litter weight was recorded in the diabetic control group (6.47±0.08), while the least was recorded in the diabetic treated group (6.24±0.12); but these changes were insignificant ($p>0.05$) when compared to control (6.36±0.11). No significant difference was recorded in the head-length and crown-rump length when litters from the experimental groups were compared to control. No physical abnormalities were observed in the litters and all litters had 20 digits. There was no cleft palate in any of the litters and no other abnormality was observed at birth, two weeks and five weeks after birth. Life-birth index was 100% for all pregnant rats.

Table 1: Pregnancy and fetal outcomes in diabetic rats treated with metformin

Groups	Control (drug vehicle)	Diabetic (drug vehicle)	Metformin (100mg/kg)	Diabetic/met (100mg/kg)
Gestation length (days)	22.5±0.96	21.8±0.28	21.7±0.64	21.6±0.53
% Weight change in pregnancy	43.6±1.32	30.4±0.98*	43.8±0.86	31.3±0.65*
No. of fetuses (d 19)	8.5±0.39	4.8±0.30*	7.5±0.39	6.5±0.40
Wt of fetuses (d 19)	6.26±0.14	6.67±0.09	6.15±0.12	5.98±0.12
Ave. litter size	8.1±0.11	4.9±0.21*	7.6±0.42	5.14±0.06
Weight of placenta (d 19)	0.04±0.01	0.06±0.02	0.04±0.02	0.05±0.01
Wt of litters at birth (gms)	6.36±0.11	6.47±0.08	6.35±0.16	6.24±0.12
Head length (cm)	1.92±0.02	1.91±0.03	1.93±0.04	1.89±0.03
Crown-rump length (cm)	5.83±0.12	6.33±0.11	6.13±0.15	6.12±0.01

*$p<0.05$ when compared to control; Bld-Blood; d-day; Glc-glucose; Met- Metformin; wt-weight

Hormone Levels During Pregnancy: Significantly elevated ($p<0.05$) levels of estradiol was observed on day 14 (d 14) and day 19 (d 19) treated non-diabetic rats [group III- 53.40±2.5 (d 14) & 56.60±5.0 (d 19)] (table 2). The remaining experimental groups did not show significant changes ($p>0.05$) when compared to the control [20.00±1.01 (d 14) & 35.00±2.5 (d 19)]. The plasma progesterone levels on d 14 was significantly elevated ($p<0.05$) in the treated non-diabetic (60.00±4.1) and the treated diabetic rats (40.46±5.7) when compared to the non-diabetic (35.00±0.6) and diabetic (30.20±2.6) controls, respectively. A significant reduction in the plasma level of CRP was

recorded on d 14 in all the experimental groups (III- 0.10±0.1 & IV- 0.14±0.11) when compared to control (group I- 0.20±0.01 & group II- 0.22±0.1). There was no significant change ($p>0.05$) observed in the plasma levels of βhCG throughout gestation in all experimental groups when compared to control.

Table 2: Effect of metformin on plasma levels of progesterone, estrogen LH and FSH in control and experimental rats

Variable	Control (drug vehicle)	Diabetic (drug vehicle)	Metformin (100mg/kg)	Diabetic/Met. (100mg/kg)
FSH (mIu/ml)	1.70±0.10	1.02±0.01*	1.25±0.30*	1.05±0.01*
LH (mIu/ml)	3.12±0.21	2.20±0.05*	2.95±0.01	2.25±0.06*
Estrogen (pg/ml)	140.00±11.40	100.00±1.83*	136.00±13.1	129.00±6.19**
Progesterone (ng/ml)	0.58±0.09	2.43±0.15*	0.70±0.11	1.45±0.19**
Fasting Plasma glc (mg/dl)	139.75±6.20	277.75±8.58*	130.84±5.11	212.75±5.97**
Fasting Plasma insulin (μIU/ml)	25.10±2.31	10.12±1.81*	23.53±6.20	9.75±1.14*
Glc:Insulin ratio	5.35	27.28*	5.56	21.82*

* $p<0.05$ when compared to control
** $p<0.05$ when compared to untreated diabetic group

Discussion

Maternal weight gain is component of stored fat, fluid gain, breast tissue, the fetus and the placenta. The highest maternal weight gain was seen in the metformin treated non-diabetic group. The number of fetuses and litter size were significantly reduced in the diabetic groups. This corresponds to studies by Erikkson; (2016), who reported decreased embryos in diabetic mothers and Ibrahim *et al*., (2002), who reported increased resorption at the uterine horns with a subsequent decrease in litter size of pregnant rats. The highest birthweight was seen in the diabetic untreated group. This corresponds to the studies of David *et al*., (2013), who studied the effect of maternal DM on the fetus and the neonate. It was reported that the increase in birthweight was due to organomegaly secondary to fetal hyperinsulinemia. Evidence exists that fetal adaptation to challenges in the intrauterine environment may adversely affect long-term cardiovascular health (Huxley *et al.,* 2007). In another observation, adaptations to a suboptimal intrauterine environment may also occur, without noticeable reduction in birthweight and still be of long-term importance (Myatt, 2004). The present experiments did not show any anomalies or malformations in the litters of all experimental groups.

During pregnancy, levels of estrogen and progesterone rise steadily, in large part as a result of placental production of these hormones. Levels of human chorionic gonadotrophin (hCG) and cortisol rise across pregnancy, reaching maximum near term and declining after delivery. The present study determined the levels of corticosterone, C-reactive protein (CRP), progesterone and estradiol, following administration of metformin during pregnancy in normal and diabetic rats. βhCG promotes the maintenance of the corpus luteum during pregnancy. Due to its high negative charge, it may repel the immune cells of the mother, protecting the fetus during the first trimester. Its level rises from conception and peaks in the second trimester, and then steadily declines to non-pregnant levels as pregnancy progresses.

Though serum levels have been shown to decrease in insulin-requiring diabetic women (Rawn *et al*., 2015), the present study did not reveal any significant changes in the level of βhCG in female diabetic rats following administration of metformin during pregnancy. CRP is a sensitive marker of systemic inflammation (Nakhishbandy & Barami, 2014). High concentrations of maternal CRP have been associated with adverse pregnancy outcomes and premature contractions may be predicted by elevated levels of CRP. Significantly lowered levels of CRP were observed in the treated groups (both non-diabetic and diabetic). This implies that metformin have both maternal and fetal protective effects during pregnancy. In pregnancy, progesterone enriches the uterus with a lining of blood vessels and capillaries so that it can sustain the growing fetus. Estradiol is a biologically active form of estrogen

produced by the placenta. Insulin stimulates ovarian and placental estrogen and progesterone secretion (Dunaif, 1992). Metformin improves on insulin resistance by increasing the peripheral effects of insulin. This increase in the peripheral effects of insulin could cause the subsequent rise in progesterone and estrogen levels recorded in the present study. Corresponding to this are findings by Shannondoah *et al*., (2002), who reported a dose dependent increase in estrogen and progesterone release by normal and diabetic placenta in vitro, following insulin administration.

In conclusion, elevated levels of progesterone and estrogen with a concomitant decrease in CRP levels during pregnancy suggest a maternal and fetal-protective effect of metformin. Findings also show that the use of metformin in pregnancy is not associated with fetal abnormalities. These suggest that metformin improves on some reproductive functions in the diabetic and even in the non-diabetic state. Hence, even though utmost care should be taken before the prescription of metformin in pregnancy, more consideration should be given to the non-hypoglycemic effects of this drug and possible use in the management of reproductive complications associated with DM.

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