Drug Discovery

Evaluation of *in utero* developmental toxicity of metformin hydrochloride in male albino rats

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ABSTRACT

Metformin is an oral antidiabetic drug of the biguanide family that is used in the management of diabetes and some forms of female factor infertility. It readily crosses the placenta and thus its safety in pregnancy is a concern. This study assessed the *in-utero* developmental toxicity of metformin in rats. Sixteen albino rats were grouped into four with each group consisting of three females and one male. The rats were allowed to mate till pregnancy. The female rats in group A received metformin throughout pregnancy, the female rats in group B received metformin in the first 13.5 days of pregnancy while the female rats in group C received metformin from day 13.5 of pregnancy till delivery. The rats received metformin by oral gavage at a dose of 300mg/kg/day. The females in the control group D did not receive the drug. The pregnant females and their litters, following delivery, were monitored till 14 days after birth. The result of the study showed that the administration of metformin during pregnancy did not result in any significant maternal or fetal developmental toxicity. Pregnant females treated with metformin showed poor weight gain and transient treatment-related side effects. There was no external fetal structural abnormalities such as abnormal tail, vertebral, cranial or facial abnormalities, severe developmental retardation or undescended testes in any of the litters. At therapeutic doses, metformin is safe in pregnant rats.

Keywords: Metformin, toxicity, developmental toxicity, congenital abnormality, rats

1. INTRODUCTION

In-utero, some harmful effects could occur in a developing organism as a result of exposure to environmental insult. Developmental toxicity refers to interference with normal development of an organism resulting from exposure of either parent before conception, the mother during conception or the developing organism after delivery to certain chemicals or conditions (Dubey et al., 2022; Hougaard, 2021). Thus,

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developmental toxicity can occur before birth or after birth. It is any reversible or irreversible, functional (physiologic) or structural (anatomic) alteration caused by environmental insult, diet, and toxic chemicals or physical factors that affect the growth, differentiation, development, or behavior of the organism (Dubey et al., 2022). Developmental toxicity studies assess to what degree a substance can interfere with normal development and cause adverse effects in the offspring when the parents get exposed to the substance.

The impact of developmental toxicity depends on the type of toxicant, the dose and duration of exposure, and the stage of pregnancy during exposure. The realization that substances can cross the placenta and inflict irreversible damage to the fetus and reproductive organs has triggered interest in developmental and reproductive toxicology (DART) in other to protect future parents and children (Hougaard, 2021). The developing organism is more susceptible to these toxicants during the early stages of *in-utero* development. Metformin is an oral anti-hyperglycemic agent from the biguanide family (Panchaud et al., 2018). Metformin has been widely used for the treatment of diabetes mellitus, ovulation induction, polycystic ovarian syndrome, ovulation induction, prevention, and treatment of gestational diabetes (Given et al., 2018; Panchaud et al., 2018). Metformin acts primarily in the liver; however, it can also act on other tissues including reproductive tissues (Tartarin et al., 2012).

Metformin may accumulate in many tissues like liver, muscle, pancreas, pituitary, hypothalamus, adipose tissue, and the gonads. Many clinicians have reluctance to recommend oral antidiabetic drugs for the management of diabetes in pregnancy because of their ability to cross the placenta and the risk of neonatal hypoglycemia (Given et al., 2018; Lindsay and Loeken, 2017). The plasma concentrations of metformin in the maternal and fetal circulation are similar (Charles et al., 2006; Lindsay and Loeken, 2017). There are concerns about potential adverse effects on the fetus when metformin is used in pregnancy because it crosses the placenta. Metformin has the propensity to cause cellular energy depletion and affect one-carbon (1-C) metabolism, hence there are concerns that there may be some long-term effects of prenatal metformin exposure on fetal programming and long-term health of exposed offspring (Priya and Kalra, 2018).

The findings of previous studies evaluating the safety or otherwise of in-utero exposure to metformin are conflicting. Hague et al., (2003), Amin et al., (2008), Rø et al., (2012) found no increased incidence of congenital anomaly or differences in anthropometric indices while Piacquadio et al., (1991), Kovo et al., (2006) and Carlsen et al., (2012) noted that *in-utero* metformin-exposed children had higher incidence of congenital anomalies and had a higher anthropometric indices like birth weight, height, etc. Therefore, further research is necessary to shed more light on this. Recommendations for the use of metformin in pregnancy are controversial.

In Scotland, metformin is recommended as first line pharmacological treatment for diabetes Scottish Intercollegiate Guidelines Network, (2014) while the New Zealand Ministry of Health recommends metformin as an option (New Zealand Ministry of Health, 2014). Conversely, the American Diabetes Association did not recommend metformin because of lack of long-term safety data for offspring (American Diabetes Association, 2018). It is necessary to ascertain the teratogenic potential of metformin to properly advise these women on the risk associated with its use in pregnancy. The study aimed to determine the developmental toxicity of metformin in rats.

2. MATERIALS AND METHODS

Animals

Sixteen (16) albino rats (*Rattus norvegicus*), four males and 12 nulliparous females, between the ages of twelve (12) and fourteen (14) weeks, and with body weights between 150g and 200g, were used for the study. These rats were obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Drugs

The metformin hydrochloride 500mg tablets produced by Hovid Pharmaceuticals Nigeria Limited (Diabetmin 500mg) were used for the study. The drug was purchased from the hospital dispensary of the Nnamdi Azikiwe University Teaching Hospital Nnewi. The tablets were ground into powder and dispersed in water. Each 500mg tablet was dispersed in 4mls of water and subsequently made up to 5mls. Each 0.1mls of this suspension contained 10mg of metformin. Other materials include cage with tags for animals, digital weighing balance (West Tune Scientific, China, Model no YP5001), measuring cylinder, feeding trough, water trough, animal feed pellets procured from the animal house of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, water, rodent oral gavage tubes (Instech Laboratories, Pennsylvania USA), vernier calipers (Medilab, India).

Routine care of animals

Sixteen albino rats were randomly grouped into four with each group consisting of three females and one male. The different females in each group were further distinguished using different colours of ink and assigned 1, 2 and 3. For example, group A had three females namely A1, A2 and A3. For acclimatisation, the animals in each group were placed together in a cage for one week. The cages were maintained at room temperature in an artificially lighted room with a 12-hr light/12-hr dark cycle. Drinking water and pellet rodent feeds were given to the animals *ad libitum*. The feeds were weighed before and after feeding the animals. The animals were weighed every week.

Mating

The animals were allowed to mate till they became pregnant. The female animals were examined every morning for clinical signs such as piloerection, dull fur, lower weight gain, and decreased locomotor activity. They were also examined for the presence of vaginal plug, which is evidence of mating. The morning of the appearance of the vaginal plug was regarded as day 0.5 post coitus. The females with the vaginal plugs were separated from the rest. The feeding and care of the animals continued all through the pregnancy. Throughout gestation, all pregnant rats were observed daily for general appearance and behavior and possible mortality and morbidity.

Drug dosage and administration

The female animals in group A, B and C were placed on metformin at a dose of 300mg/kg/day. This metformin dose in rodents is needed to obtain a similar therapeutic effect in diabetic animals as in humans (Foretz et al., 2010; Wilcock and Bailey, 1990). The body weights were measured and recorded every week and the drug doses adjusted according to the body weight. The medication was dispersed in the measured volume of water in a test tube as described above. Each animal was given the calculated volume and quantity of the drug according to its body weight using the oral gavage tube and 1 ml syringe. Group A received the calculated metformin dose from day 0.5 of pregnancy until delivery. Group B received the calculated metformin dose from day 0.5 of pregnancy until day 13.5.

From day 13.5 until delivery, rats in group B received water. Group C received metformin from day 13.5 until delivery. From day 0.5 until day 13.5, the rats in group C received water. The female animals in the control group D did not receive the drug but received water from day 0.5 of pregnancy until delivery (Table 1). The female rats in each group were further identified using the numbers 1, 2, and 3. Thus, in group A, there were A1, A2 and A3. The drugs or water were administered to the animals daily during the specified period using a cannula. The first 13 days of gestation in rats corresponds to the first trimester in humans (Tartarin et al., 2012). This is the sensitive period for the development of the testis since the formation of the seminiferous cords, as well as the proliferation and apoptosis of germ cells occur at this time.

Table 1 Schedule for Administration of Metformin

Group	A	B	C	D
Day 0.5 to 13.5	Metformin	Metformin	Water	Water
Day 13.5 till Delivery	Metformin	Water	Metformin	Water

Litter characteristics and anthropometry

Following delivery, the length of gestation, litter size and birth weights were recorded. The numbers of stillbirths and live pups were noted. The day of birth was designated as post-natal day (PND) 0. Morphological abnormalities such as abnormal tail, vertebral, cranial, or facial abnormalities or severe developmental retardation were looked out for. Rearing of the pups continued until postnatal day 14. The weight of the male pups was measured.

Statistical analysis

The data was checked for completeness and tabulated. The data was analysed using the Statistical Product and Service Solutions (SPSS) computer software version 26.0 (IBM Corporation). All values were expressed as the mean ± S.D. of the four groups. Data were analysed using one-way ANOVA to test for significant differences among groups. Statistical significance was inferred at p-value ≤0.05.

3. RESULTS AND DISCUSSION

Maternal toxicity

The maternal findings for the mated females treated with metformin by gavage during the entire period of pregnancy are presented in Tables 2, 3, and 4. There was no significant difference in the maternal pre-pregnancy body weights; however the weight gain during the entire period of pregnancy was significantly reduced in groups A and C (which received intervention in the later part of pregnancy) compared to the treatment group B, or the control group D. (Tables 2 and 3) Amon0g the rats in the treatment group, the mean weight gain was lowest in group A and highest in group B. The weight gain in this study is a complex relationship as it may be related to the number of fetuses, the birth weight of the fetuses, the effect of the drugs on the rats or the physiologic changes of pregnancy.

The suppression or decrease in the body weight gain in the exposed rats could be a feature of significant maternal toxicity of metformin. Even in non-pregnant rats, Adaramoye et al., (2012) have reported a reduced weight gain in rats treated with metformin compared with controls. This discrepancy in weight gain may also be related to the litter characteristics (number and weight) per dam rather than the toxic effect of metformin. For example, the litter size per dam is highest among the control compared with the exposed animals. Similarly, the birth weight of the litters was highest in the controls group compared with the test groups. These factors may have contributed to the higher weight gain in the control group than in the treatment group.

Table 2 Average Weight of the Rats during the Study

Group	A	B	C	D
Weight(g) at Day 0	217.7±7.1 (3)	217.3±6.5 (2)	220.7±6.7 (3)	219.3± 7.5 (2)
Weight(g) at Day 20	315.0 ± 5.0 (3)	325.0 ± 4.2 (2)	321.7 ± 6.4 (3)	326.5 ± 2.1 (2)
Pregnancy Weight Gain (g)	97.3 ± 12.1 (3)	107.7 ± 13.4 (2)	101.0 ± 6.1 (3)	107.2 ± 2.8 (2)
Duration of Pregnancy (Days)	21.7 ± 1.2 (3)	21.5 ± 0.7 (2)	22.0 ± 1.0 (3)	22.5 ± 0.7 (2)

Table 3 Abnormal clinical signs noted on pregnant rats. (A = Absence, P = Presence)

Group	A			B			C			D		
	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
Piloerection	A	P	A	P	A	A	P	P	A	A	A	A
Dull Fur	P	A	A	A	P	A	A	A	P	A	A	A
Decreased Locomotor Activity	P	A	P	P	A	P	P	P	A	A	A	A
Poor weight Gain	P	P	A	P	P	P	A	P	P	A	A	A
Pregnancy Failure	A	A	A	A	A	-	A	A	A	A	P	A

Except for one female that died in group B (B3), all other females survived in both control and treated groups throughout the study. Mated females of the treatment groups (A, B and C) showed treatment related clinical signs such as piloerection, dull fur, lower weight gain, decreased locomotor activity (Table 3). All dams delivered at gestational day (GD) 21 or 22 (Table 2). There was no significant difference in the gestation length among the dams in the treatment and control groups. The number of pups and proportions of males/females did not differ in the groups (Table 4). Pregnancy failure, that is, total absence of any implantation site, was observed in 1 animal in group D (D2). The abnormal clinical signs on pregnant rats, such as piloerection, dull fur, lower weight gain, decreased locomotor activity etc are not uncommon findings in animal studies especially in the first few days of administration of the medication.

Table 4 Litter Characteristics at birth and at Day 14

Group	A	B	C	D
LITTER SIZE				
Total at Birth (Mean)	19 (6.3)	16 (8.0)	20 (6.7)	18 (8.5)
Live litter	17	15	18	16
Stillbirth (Stillbirth rate (%))	2 (10.5)	1 (6.3)	2 (10)	2 (11.1)
Live litter size at Day 14	14	11	14	12
Male litter at Day 14	8	5	6	7
LITTER WEIGHT (g)				
Mean weight at birth	4.9 ± 0.2	5.0 ± 0.4	4.8 ± 0.2	5.2 ± 0.3
Mean weight of males at Day 14	20.9 ± 2.1	21.8 ± 1.8	21.6 ± 2.0	22.8 ± 2.3

Post Hoc analysis of weight of all litters at birth:

A vs B =1.000, A vs C = 1.000, A vs D = 0. 065,

B vs C = 0.085 B vs D =0.068, C vs D =0.062

Post Hoc analysis of weight of male litters at Day 14:

A vs B =0.083, A vs C = 0.1000, A vs D = 0. 065,

B vs C = 1.000 B vs D =0.083, C vs D =0.626

These changes may be due to the adaptive response to the drug. Similar study by Kim et al., (2001) reported similar abnormal clinical signs. There is no evidence that the death of one female in group B is the effect of the administered medication. It could be a random event. This is because none of the other animals similarly exposed to the drug experienced this. Rats in group A received treatment all through pregnancy, yet no death was noted. Despite the reported stillbirth, it is important to note that no surviving litter had any external fetal morphological abnormalities such as abnormal tail, vertebral, cranial or facial abnormalities, severe developmental retardation or undescended testes.

Developmental Toxicity

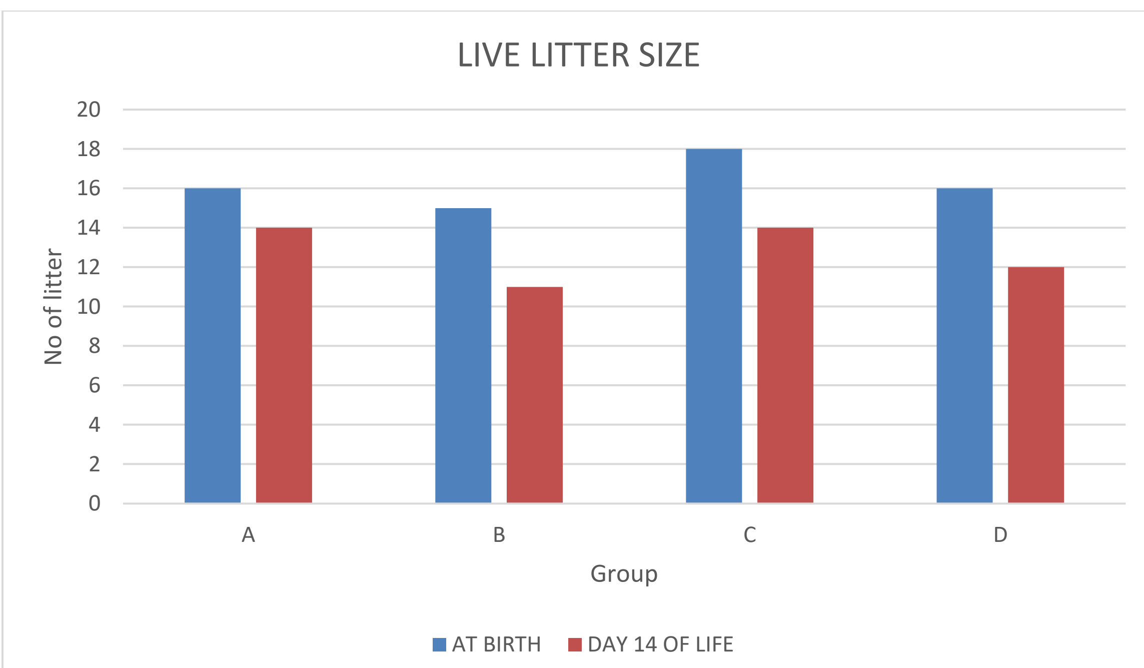

Figure 1 Live litter size at birth and on day 14

The stillbirth rate was higher in the control group (11.1%) compared with the treatment groups which had rates of 10.5% in group A, 10% in group C and 6.3% in group B (Table 4). The birth weights of the litters were higher in the control group than in the treatment groups. This discrepancy was also maintained in the weight of the male litters on post-natal day 14 (Table 4). There were no cases of fetus with abnormalities such as abnormal tail, vertebral, cranial or facial abnormalities, severe developmental retardation or non-descent of testes in any of the groups. There was no difference in the number of live litters in the groups. The number of litters that died before two weeks was not significantly different in the treatment and control groups. The number of live fetuses per litter in the treatment groups was lower than in the control group. One (1) female rat died in group B while there was pregnancy failure in 1 female rat in group D leaving these two groups with two female rats that littered. The number of live litters after two weeks was not significantly different in the treatment and control groups (Figure 1).

4. CONCLUSION

Metformin is commonly indicated in pregnant women due to gestational diabetes mellitus and other indications. The findings showed that administration of metformin during pregnancy did not result in any significant maternal toxicity. Similar effects were noted irrespective of the period of pregnancy when the drug was administered. Maternal body weight gain during pregnancy was suppressed, though not significant, in the treatment groups. The decrease in the weight gain in the exposed rats may be related to the litter characteristics (number and weight) per dam rather than the toxic effect of metformin. The females in the treatment groups showed treatment related clinical signs such as piloerection, dull fur, lower weight gain, decreased locomotor activity. It is important to note that in this study, metformin administration at any stage in pregnancy did not induce any external fetal structural abnormalities such as abnormal tail, vertebral, cranial or facial abnormalities, severe developmental retardation or undescended testes in any of the groups.

Rodents closely resemble human in terms of enzyme system and drug metabolism and this is why they are frequently used in preclinical research. The findings of this study will serve as an impetus for a human study. We recommend a clinical study to investigate the developmental toxicity of metformin on humans since findings from animal studies do not always correlate directly with clinical findings. Our finding suggests that at therapeutic doses, metformin does not cause developmental toxicity. This will help in counselling and alleviating the anxiety of prospective mothers who discovered that they were pregnant while still taking metformin. If a human study confirms the safety of metformin from causing developmental toxicity, metformin will be an alternative to insulin in the management of diabetes in pregnancy.

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Contribution to Authorship

Okoro CC and Afonne OJ participated in conceptualization and designing of the study. Okoro CC and Ifediba EC participated in data collection and data analysis. Afonne OJ supervised the research. All authors contributed to manuscript writing and revision, gave final approval of the manuscript to be published and agreed to be accountable for all aspects of the work.

Statement of Ethical approval

This study received the approval of the Animal Research Ethics Committee of Nnamdi Azikiwe University Awka with the approval number: NAU/AREC/2023/00066. The study was conducted according to the University's ethical principles for Animal research.

Informed consent

Not applicable.

Conflicts of interests

The authors declare that there are no conflicts of interests.

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Data and materials availability
All data associated with this study are present in the paper.

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