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Examining Effects of Metformin on Live Birth Rate in PCOS-Induced Mice: A Research Protocol

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Abstract

Introduction: Polycystic Ovary Syndrome (PCOS) is the prevailing endocrinopathy of women of reproductive age. With insulin resistance as a key feature of the disorder, metformin has been observed to improve ovulation induction, anovulatory infertility, and hyperandrogenic symptoms. Some hesitancy to prescribe this medication can be attributed to its possible contribution to pregnancy loss. This research protocol investigates the effect of metformin therapy on live birth rates (LBRs) to deduce its efficacy extending beyond fertility for pregnancy success. Congenital anomalies and birth weights are also evaluated.

Methods: A transgenic C57BL/6 mouse model is adopted using the bovine luteinizing hormone beta subunit (LHβ) gene to induce a high expression of LH and subsequently lead to PCOS-like symptoms. At first anovulation detection, metformin therapy is administered to the experimental mice. At 26 days, artificial insemination is conducted to induce pregnancy. The experimental mice are divided into three sections: the first terminating metformin treatment at fertilization (MetF), the second at the end of the first trimester ($MetT_1$), and the last extending throughout pregnancy ($MetT_2$).

Results: It is hypothesized that $MetT_2$ mice will yield a higher LBR than those without continuous metformin treatment, with the MetF group producing the lowest rate of all mice provided with the drug. Furthermore, the control group should noticeably differ in LBR compared to the experimental group. Congenital anomalies and birth weights are expected to remain unchanged regardless of treatment.

Discussion: The currently available information regarding metformin's influence on LBR is inconsistent, but it is reasonable to conclude that there will be some improvement. Fetal outcomes have been less explored.

Conclusion: By comparing LBRs, congenital anomalies, and birth weights, this experiment can expose the most advantageous duration of metformin administration. Future directions should include combining with other pharmacological therapies and investigating metformin effects on LBR using different animal models.

Keywords: PCOS; metformin; ovulation induction; fertility; pregnancy; congenital anomaly; birth weight; transgenic mouse model; C57BL/6 mice

Introduction

Polycystic Ovary Syndrome (PCOS) is the most common endocrine disorder affecting 4 to 20% of reproductive-aged women [1-7]. Diagnosis of PCOS is commonly made using the Rotterdam criteria, which requires two out of the three clinical features of hyperandrogenism, oligo-anovulation, and ovarian morphology to be demonstrated [7-9]. Common symptoms of PCOS include endometrial hyperplasia, enlarged ovaries, irregular menses, pelvic pain, acanthosis nigricans, hirsutism, alopecia, and subfertility [10,11]. PCOS women experience fertility challenges and are prone to higher risks for preeclampsia, gestational diabetes, and preterm birth compared to healthy women [12-14].

The etiology of this condition remains unclear due to the multifactorial nature of the disorder, though studies have suggested that genetics, heritability, lifestyle, obesity, insulin resistance, and excessive luteinizing hormone (LH) stimulation at puberty play a role in PCOS development [1,7,11,15]. Hormonal imbalances are also implicated in the pathogenesis and pathophysiology of this disorder. Gonadotropin-releasing hormone (GnRH) levels are observed to be elevated in women with PCOS, consequently leading to augmented androgen production that may promote the presentation of hirsutism, alopecia, and acne [10,16-18]. GnRH also influences the production ratio of LH to follicular-stimulating hormone (FSH), supplementing the production of LH [5,16]. The ensuing reduced FSH levels can disrupt follicular maturation and impact ovulation [5,19].

Challenges with fertility are highly prevalent in PCOS patients, occurring in 70 to 80% of cases [20,21]. Treatment

commonly starts with preconception care, including lifestyle changes and folic acid therapy [21,22]. Infertility can further be circumvented with clomiphene citrate (CC), which is considered the first-line pharmacological intervention for ovulation induction [7,23,24]. This drug has been shown to induce successful pregnancies in 20 to 40% of PCOS patients; however, 15 to 40% of women with PCOS are resistant to CC [10,25-28]. Therefore, other ovulation induction agents like letrozole and metformin are explored as treatment options and administered in conjunction with CC or by themselves [7].

Metformin, a common insulin-sensitizing drug, has been shown to have significant benefits in managing PCOS symptoms, including irregular menses, elevated androgen levels, and pregnancy risks [24,29-32]. This may be attributed to the relationship between insulin resistance and PCOS [24,33]. Insulin activity in patients with the disorder may be influenced and inhibited by the development of hyperandrogenism [17,33]. Meanwhile, insulin resistance and subsequent hyperinsulinemia can affect hormones and reproduction, causing several PCOS characteristics [17,33,34]. The AMP-activate protein kinase complex participates in insulin signalling, energy glycolysis, and lipogenesis, and can even be activated by metformin to combat this insulin resistance – thus helping PCOS patients [35-37].

Oligo-anovulation, and therefore subfertility, may be improved with the drug-induced decrease in insulin levels circulating in the body [24,31,35]. Several studies have shown that metformin reduces early pregnancy losses and increases live birth rates (LBRs) during pregnancy [30,34,38]. Naturally, other articles have contradicted these findings, showing no effect from drug use [39,40]. Considering the abundance of research that points towards an improved change in pregnancy success, the former conclusion is accepted. This research protocol adopts an animal model to investigate the effect of continued metformin use throughout pregnancy and LBR. It is hypothesized that the longer the duration of the drug administration, pregnancy success is increased. Because there is limited research pertaining to the relationship of metformin with fetal development, all data concerning congenital anomalies and birth weights are also collected to investigate the fetal response to the drug. There is no expected impact of metformin on these characteristics based on insufficient previous reporting on unfavourable fetal outcomes.

Methods

1. Animals

C57BL/6 mice are housed in standard animal cages in a temperature-controlled room (22 ± 1°C) and supplied with unlimited access to water. They are held under a 12-hour light: 12-hour darkness cycle and monitored daily for wellness, signs of cage aggression, and disease. A controlled chow diet is provided to prevent obesity. Before induction of PCOS, weight is analyzed. If mice are obese (> mean + 3 stdev), mice are excluded from the study due to hormonal and metabolic changes that significantly affect fertility and pregnancy success [31]. All mice are euthanized after the experiment following Canadian Council on Animal Care guidelines.

2. PCOS Induction

Induction of PCOS will be done in accordance with methods in Proceedings of the National Academy of Sciences in 1995. Genetic induction of PCOS is conducted by increasing LH secretion from the pituitary through the expression of an LHβ subunit transgene, modified by a bovine α-subunit promoter [41]. A short fragment containing the C terminus of the bovine LHβ is fused with the C-terminal peptide (CTP) and lengthened to include the entire bovine LHβ cDNA fused to the CTP [41]. The procured transgene will be microinjected into the fertilized oocytes [41]. PCOS induction in the mice is then confirmed with blood analysis, and secondary verification is provided by the presence of two characteristics from adapted Rotterdam criteria: hyperandrogenism, abnormal estrous cycles, and increased presence of antral follicles.

3. Pregnancy Induction

Pregnancy in mice is induced via artificial insemination. Testes are extracted from healthy sexually mature male C57BL/6 mice, and sperm are isolated from the epididymis and vas deferens into a milk solution [42]. The milk mixture is inserted into the reproductive tract of the transgenic female mice during ovulation [42]. Artificial insemination commences at the onset of the reproductive period and estrous cycle, approximately 26 days after birth [43]. The procedure is repeated every day for five days to increase the probability of fertilization. Pregnancy success is primarily measured by the number of live births. The presence and nature of congenital anomalies and irregular birth weights are also considered.

4. Experimental Protocol

Metformin is purchased from Spectrum Chemical Corp. (New Brunswick, NJ, USA) and administered daily (300mg/kg orally) via a cannula. The mice are randomly divided into four treatment groups. The control group comprises mice that do not receive metformin treatment. Meanwhile, the experimental groups receive drug treatment: metformin treatment terminating at fertilization (MetF), metformin treatment terminating at the end of the first trimester ($MetT_1$), or metformin treatment extending throughout the pregnancy ($MetT_2$). The gestational stages of mice are different and shortened compared to humans; the first trimester involves gestational days 1-10, the second trimester involves gestational days 10-20, and the third trimester occurs after birth as the “brain growth spurt” [44].

5. Blood Analysis

Blood samples are drawn from the venous and arterial blood vessels of mice. Testosterone, FSH, and LH levels are investigated through hormone testing as a secondary outcome of this experiment. Confirmation of PCOS induction is obtained from an increased presence of testosterone and LH. Increases in progesterone levels are also sought after to demonstrate pregnancy induction. Blood analysis is implemented at fertilization, each trimester, and after birth.

6. Statistical Analysis

Multivariate Analysis of Variance (MANOVA) is selected to compare the LBRs of all groups. It is also used to investigate differences in birth weights and for blood analysis. Tukey's Multiple Comparison test is applied after MANOVA to further verify statistical differences among the control and experimental groups. The nature of the congenital anomalies, as well as the number of occurrences, are assessed independently.

Results

PCOS induction is confirmed with hormone testing via blood analysis. Elevated levels of testosterone and LH indicate successful induction of PCOS. Two out of three clinical features characterizing the adapted Rotterdam criteria are to be demonstrated: hyperandrogenism, abnormal estrous cycles, and excess numbers of antral follicles. Pregnancy is detected with increased serum progesterone levels.

Metformin use is hypothesized to yield discernably higher LBRs than the control group. For the experimental groups, $MetT_2$ mice are expected to have the most elevated LBR and, therefore, experience greater effects from metformin. Clinical features like endometrial hyperplasia and ovarian size are also expected to diminish. Testosterone and LH: FSH levels of the $MetT_2$ mice would be the most decreased compared to the control, MetF, and $MetT_1$ groups. The second highest LBR should be generated by $MetT_1$ mice. The number of congenital anomalies and birth weights that fall out of the normal range should not be affected by metformin treatment duration. Statistical significance of each outcome between the control and experimental groups can be concluded from the MANOVA test with a p-value less than or equal to the significance level of 0.05. Using Tukey's test, two groups can be deemed significantly different when the q_s value is larger than the critical value q_a.

Discussion

With metformin treatment, there is an expected increase in the LBR. Meanwhile, a lack of change in congenital anomalies and irregular birth weights compared to the control group is also anticipated. For the secondary outcomes, there is a significant reduction in testosterone and LH: FSH levels with metformin administration. This effect is demonstrated to be more profound with a longer duration of metformin.

Several studies have assessed the influence of metformin on LBRs, but no consensus is clear. One systematic review sought to evaluate the impact of insulin-sensitizing drugs on the metabolic and reproductive features of PCOS [34]. Their findings suggest that metformin alone benefits live birth compared to the placebo [34]. However, their evidence quality was low, and the statistical difference was not substantial [34]. Similarly, another systematic review noted a marginal improvement in LBRs with metformin [45]. A systematic review with meta-analysis of randomized controlled trials (RCTs) focused on the use of metformin on PCOS patients that received gonadotropins for ovulation induction [46]. They discovered a significant effect represented as double the LBR [46]. Pregnancy rate was likewise revealed to have improved by more than two-fold [46]. However, the evidence presented is also based on low studies' quality [46]. One meta-analysis dealt with several limitations when evaluating pregnancy rates, with many trials not delineating it as an outcome measure or implementing a control for external causes of infertility [39]. Regardless of these limitations, they had deduced a lack of evidence for the benefit of metformin in the clinical pregnancy rates [39]. Instead, a significant treatment effect was found with the conjunction of metformin and CC compared to CC alone [39]. No difference in the LBR between the metformin and control groups was again seen in a separate meta-analysis of RCTs [40]. This finding remained despite post hoc subgroup analysis based on BMI [40]. Overall, changes to the LBR with metformin are achievable, though the extent may not be significant. The drug has been reported to produce better results as an amplifier of other pharmacological treatments.

Fewer studies have explored the influence of metformin administration on congenital anomalies and birth weights, though their findings are more homogeneous. The skewed focus on ovulation, pregnancy rates, and LBRs instead of birth defects was recognized in one meta-analysis [47]. As a result, they found they were limited due to their need to extrapolate from those studies that were not designed to assess congenital anomalies [47]. Despite this, they were able to conclude that there is no evidence of an increased risk of major birth defects [47]. Similarly, a narrative review that primarily analyzed the administration of metformin in pregnant women with gestational diabetes mellitus had also observed that most studies discussing the drug's effect on the frequency of offspring malformation and deformity concluded no increase [48]. Birth weights were likewise revealed to be unaffected by metformin treatment in a systematic review that compared individual patient data from RCTs [49]. Therefore, existing studies suggest that metformin is unlikely to influence the presence and nature of congenital anomalies and irregular birth weights.

As shown by some of the previously described studies, it is possible that no difference will be found between the control and treatment groups. Random and systematic errors can cause this, or the hormonal reaction to metformin may simply not be strong enough to affect pregnancy success. In the case that metformin does produce a change in the LBR, a lack of drug sensitization may transpire and lead to no variation in the data across all treatment groups.

The internal validity of the data that describes the causal effect of metformin is improved by the identification of controlled variables, including temperature, diet, and metformin concentration and administration. Furthermore, the easy replicability of this experiment provides the opportunity to verify the results. Being an animal study can be a strength as well. With the chance of congenital anomalies and abnormal birth weights, it is more ethical to experiment on mice. It is additionally unlikely that an appropriate sample size can be gathered of women who either consent to being inseminated for an experiment or be introduced to a drug that can impact their neonatal outcomes. Finally, this is the first experiment that compares the LBRs of various metformin therapy durations in one study.

The limitations of this study must also be acknowledged. First and foremost, the practical applications of the research findings rely on translational work from animal studies to human studies. Despite the congruity of reproductive tract development in mice and humans, anatomic, developmental, and endocrinologic differences should be considered [50-51]. The selected mouse model resembles PCOS with the presence of increased LH, testosterone, estradiol, and insulin levels along with the prolonged luteal phase, infrequent ovulation, infertility, and pathological ovarian changes [41,52]. This study targets that elevated hormonal activity and infertility as well as monitors the clinical features. Nevertheless, it is not authentic PCOS. While hyperinsulinemia is present in the animal model, insulin resistance is not, which poses a risk to the response to metformin, considering insulin activity is acted on by the drug [24,34-35]. Lastly, sample size is always a crucial factor to consider, with influences on statistical power and reliability. Since not all female mice are likely to have successful inseminations, the sample size may be reduced.

Conclusions

The LBR of PCOS-induced mice is expected to increase with a greater extent of metformin treatment. This study's findings are crucial to understanding when to terminate metformin administration for ovulation induction. Similarly, this information can be clinically relevant for pregnancies with type 2 diabetes where this drug is consumed. The relationship between metformin and pregnancy success may be further explored by investigating combined treatments such as with CC or letrozole. For better animal-to-human translation, larger animal models may be used.

List of Abbreviations Used

CC: clomiphene citrate
CTP: C-terminal peptide
FSH: follicular-stimulating hormone
GnRH: gonadotropin-releasing hormone
LBR: live birth rate
LH: luteinizing hormone
MANOVA: multivariate analysis of variance
MetF: metformin treatment ending at fertilization
$MetT_1$: metformin treatment ending after the first trimester
$MetT_2$: metformin treatment ending after the second trimester
PCOS: polycystic ovary syndrome
RCT: randomized controlled trial

Conflicts of Interest

The author declares that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

Ethics approval is required if the experiment is executed. The exclusion of human trials implies that participant consent is impractical.

Authors' Contributions

SMB: contributed to study design and planning, drafted the manuscript, and gave final approval of the version to be published.

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