RESEARCH ARTICLE

Comparative toxicological analysis of metformin (Biguanide) and glibenclamide (Sulfonylureas), using zebrafish embryotoxicity test (ZFET)

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ABSTRACT

Background and Objective: Type 2 (T2DM) and gestational diabetes mellitus (GDM) among pregnant Filipinos have been increasing over the years because of lifestyle westernization. While insulin has been the safe mainstay when dietary measures fail to maintain normoglycemia during pregnancy, recent studies have suggested that oral hypoglycemic agents (OHAs) such as metformin and glibenclamide, may offer cheaper and efficacious alternatives. The problem, however, is the passage of these drugs through the placenta which may pose possible danger towards the development of the growing embryo. The proposed study aimed to evaluate and compare the embryotoxic and teratogenic potentials of the varying concentrations of the two PhilHealth covered oral hypoglycemic agents in the Philippines, namely, metformin (biguanide) and glibenclamide (sulfonylureas).

Methodology: In this study, a comparison of embryotoxic potentials of metformin and glibenclamide was conducted using zebrafish embryotoxicity test (ZFET) across concentrations found in fetal (10, 20, 100, 500, 1000, 2000 µg/L) and maternal serum (10, 20, 100, 500, 1000, 2000 mg/L).

Results and Conclusions: Results revealed that metformin showed no significant ($p>0.05$) lethal effects, but revealed significant risk for teratogenicity, specifically decreased head and tail lengths and advanced hatching. Conversely, glibenclamide revealed significant potential for lethal (*e.g.*, coagulation) and teratogenic effects including pericardial and yolk sac edema, spinal deformity, and increased tail length. Comparative evaluation between the two OHAs revealed that glibenclamide has significantly ($p<0.05$) higher lethal and teratogenic effects. Together, results suggest that the use of metformin over glibenclamide is favorable for safety testing in pregnant women suffering from T2DM and GDM for the benefit of expanding treatment options for these diseases.

Keywords: *embryotoxicity, glibenclamide, metformin, teratogenesis, zebrafish, oral hypoglycemic agents*

Introduction

The prevalence of Type 2 diabetes mellitus (T2DM) in pregnancy and gestational diabetes mellitus (GDM) in the Philippines has risen to over 14% [1] and has been observed to progress at a rate of 0.6% per decade as a probable result of lifestyle westernization [2]. Moreover, observational studies have shown that a higher incidence of embryopathy is consistently observed among offspring of mothers with T2DM and GDM than among offspring of non-diabetics [3].

The mainstay for treatment of T2DM in pregnancy and GDM continues to be Maternal Nutritional Therapy and human insulin, a US FDA approved system established and recommended for its practical implication and safety for both the mother and child. Although insulin therapy has been the gold standard and a universally accepted mode of treatment over several decades, it is considered labor-intensive, especially in the context of developing countries, where limited resources, poor logistics, and poor literacy rate are still a hard realities. Moreover, needle phobia, multiple daily injections, potential for hypoglycemia, weight gain and psychosocial stigma, and gender discrimination, perhaps, makes injectable therapy, somehow cumbersome for many pregnant patients [4,5]. Thus, the recommended therapy by injectable insulin along with customized diet and exercise have been eliminated as affordable options for the pockets of the general public. As a consequence, a demand for the

research of an alternative has led to the exploration of oral hypoglycemic agents, metformin and glibenclamide. By theory, oral hypoglycemic drugs provide a less invasive and efficacious alternative that achieves similar perinatal outcome while enhancing patient compliance [6]. Additionally, these two medications are the only two medications covered by PhilHealth, Philippines' national health insurance program; hence, they are significantly cheaper than insulin [2].

Metformin (N, N-dimethylimidodicarbonimidic diamide hydrochloride) is a first-line therapy for type 2 diabetes mellitus, and is one of the most commonly prescribed drugs worldwide [7]. Metformin acts primarily at the liver by reducing glucose output and, secondarily, by increasing insulin sensitivity and, consequently, glucose uptake in the peripheral tissues [8].

On the other hand, glibenclamide (or glyburide), is an antidiabetic drug in a class of medications known as sulfonylureas, closely related to sulfonamide antibiotics [9]. This drug acts to increase secretion of insulin from the pancreas, probably by interacting with sulfonylurea receptors on beta cells or by binding and inhibiting the ATP-sensitive potassium channels (KATP) in pancreatic beta cells [10,11].

Observational studies by Schwartz *et al.* [12] indicate that glibenclamide passes freely through the placenta and that the fetus is exposed to levels comparable with therapeutic concentrations in adults, much like metformin. Despite the reported efficacies of these oral hypoglemic drugs, both have been associated with the occurrence of congenital malformations in animals [9,13,14,15].

Danio rerio (zebrafish) is a species that has been greatly favored by scientists for use in studying vertebrate biology. The use of zebrafish in genetic studies is advantageous since scientists have amassed a great deal of understanding of the fish's genetic composition [16]. In 2013, a complete genome sequence of *D. rerio* was eventually published [17]. Furthermore, around 84% of genes related to human disease have a counterpart in zebrafish [18]. The use of zebrafish has been extended for testing pharmaceuticals for toxicity much like that of Hallare *et al.* [19] testing the safety of diclofenac. The microplate-based quantitative assays can be performed in a similar fashion to cell-based assays to provide an *in vivo* assessment of compound effects at an earlier stage in drug discovery. The optical clarity of the zebrafish embryo, its small size, relatively cheap maintenance, high fecundity, and rapid development makes the zebrafish embryo a suitable model organism for high-throughput assay. These ideal characteristics make zebrafish a useful preclinical model organism for predicting drug toxicity in humans [20].

This study attempted to evaluate and compare the embryotoxic and teratogenic potentials of varying concentrations of two PhilHealth covered oral hypoglycemic agents in the Philippines, namely metformin (biguanide) and glibenclamide (sulfonylureas). More specifically, it aimed (1) to determine and compare the percent mortality of zebrafish (*D. rerio*) embryos to varying concentrations of metformin and glibenclamide, (2) to determine and compare the percent teratogenicity of zebrafish (*D. rerio*) embryos to varying concentrations of metformin and glibenclamide, and (3) to determine and compare the mean head and tail lengths of zebrafish (*D. rerio*) embryos to varying concentrations of metformin and glibenclamide.

Methodology

Preparation of solution and ZFET

Metformin and glibenclamide were purchased from Sigma-Aldrich. Different concentrations of metformin and glibenclamide were prepared by dissolving in 1% dimethyl sulfoxide (DMSO) through serial dilution of the highest concentration to the lowest. These solutions were brought to the laboratory for comparative assessment of the embryotoxic and/or teratogenic potentials of the different concentrations of metformin and glibenclamide, respectively. Specifically, the percent mortality, lethal, sublethal endpoints, head and tail length measurements were observed and compared using the zebrafish embryotoxicity (ZFET) test according to Hallare *et al.* [19] with modifications on exposure scheme and toxicological endpoints. Additional protocols regarding the maintenance of the zebrafish were done according to the OECD Guideline for the Testing of Chemicals [21].

Population and sampling technique

The experimental setup was accomplished in triplicate having a total sampling population of 30 embryos (*n=30*) subjected to treatment per concentration of metformin and glibenclamide. For each trial, preliminary exposure of a total of 15 randomly selected healthy embryos was completed and that 10 out of 15 pre-exposed viable embryos were selected randomly to be subjected to the different treatments per dilution. By using the random sampling technique without replacement, the possibility of other variables to interfere with the normal development of the embryos was minimized,

thus, any abnormalities in the developing embryos can be attributed to the effect of the treatment concentrations.

Parental zebrafish procurement and maintenance

Parental sexually mature zebrafish (*D. rerio*; age: 4-6 months old), 20 males and 20 females, were obtained from the BFAR-NFFTC fish farm of the Central Luzon State University located in Muñoz, Nueva Ecija, Philippines. The fish were transported using Calypso™ fish transportation bags saturated with oxygen to the aqua-toxicological laboratory of the University of the Philippines Manila. Male and female *D. rerio* adults were kept separately into two 20 L (41 × 25 × 20 cm) glass aquaria. Both aquariums were filled up to only three-fourths of its capacity with dechlorinated tap water by addition of 5 mL Sea Quest Aqua Product™ dechlorinating solution for every 10 gallons of tap water, and the following conditions were maintained at feeding time every day: 26 ± 1°C (temperature), 379 mg/L $CaCO_3$, 21.3 dH (hardness), 744 µS/cm (electrical conductivity), 8.2 ± 0.2 (pH), 10.5 ± 0.5 mg/L O_2 (dissolved oxygen), and a 12-h dark/12-h light photoperiod using 18-watt energy-saving LED tube lights.

Aquarium filters and aeration pumps were supplied to provide a continuous oxygen supply and a constant filtering system in the glass aquarium. Activated carbon granules were installed in the aquarium filters for better filtering. The zebrafish were fed twice daily with commercially available artificial diet (*TetraMin*™ flakes) once at 7AM and once at 7PM. Aquarium filter compartments were cleaned once every two weeks to remove accumulating residual zebrafish waste and excess food. Replacement of 20% of the water in the aquarium was done every 48 hours with minimal distraction to avoid stressing of the zebrafish.

Zebrafish spawning and egg collection

On the evening before spawning, collection of eggs was done by laying down a rectangular stainless tray box at the bottom of a separate aquarium, which served as a spawning chamber. The sexually mature zebrafish, 6:4 male-female ratio were placed over the submerged 25 × 15 × 18 cm rectangular mesh wire box with a grid size of 2.5 mm to prevent egg predation during mating and spawning [22]. Spawning and fertilization were triggered once the light was turned on and were completed within 30 min. The mature zebrafishes used for spawning were separated and fed from another aquarium. The eggs were collected randomly from the spawning chamber by suction using a micropipette with a widened opening to avoid possible egg abrasion and for easier suction. These were emptied into Petri dish plates pre-saturated with reconstituted water to be rinsed several times.

Toxicant and exposure procedures

To start exposure with minimum delay, at least twice of the number of fertilized eggs needed per treatment group were randomly selected. These were immediately transferred into petri dish plates containing the varying concentrations of metformin and glibenclamide in DMSO, as well as the positive and negative control not later than 60 min post fertilization. Each petri dish plate contained a total sampling population of 30 embryos (*n=30*; 10 eggs per replicate) subjected for pre-exposure to each of the thirteen concentrations (0, 10, 20, 100, 500, 1000, 2000, 10000, 20000, 100000, 500000, 1000000 and 2000000 µg/L) of metformin and glibenclamide, respectively, using DMSO [19]. Reconstituted water (based on ISO 6341) served as the negative control. The selection of 5% EtOH as a positive control was decided based on previous screening tests carried out in the laboratory, showing very low to no mortality in 1-2% and moderate mortality in 3-4 % EtOH.

Through a stereomicroscope (*Leica ES2*), fertilized eggs undergoing cleavage and showing no obvious irregularities during cleavage (*e.g.*, asymmetry, vesicle formation) or injuries of the chorion were selected and considered viable and were separated from unfertilized eggs. Viable eggs at this stage have 128 blastomeres, which is a round mass of cells attached to the yolk. After 2 hrs of pre-exposure, viable zebrafish eggs were randomly collected from the primary exposure chambers along with 0.1 mL of the test solution and were transferred to 96-well plates pre-saturated with 0.2 mL of the test solution of varying concentrations for 24 hrs using a micropipette with a widened tip. For both well-plates, 10 wells of each row contained 0.3 mL of a certain concentration. Occasional stirring as well as replacement of the medium were done daily to ensure even distribution of the chemical. Hence, a total of approximately 900 eggs were used in the study: 2 types of oral hypoglycemic agents (metformin and glibenclamide) × 15 treatments (13 concentrations + 2 laboratory controls) × 3 replicates × 10 eggs.

Qualitative embryotoxicity bioassay

Embryotoxicity (percent mortality and occurrence of lethal endpoints) and teratogenicity (occurrence of sublethal endpoints) were evaluated using the digital microscope (*Olympus*™ MIC-D Digital Microscope) at specified time points (t = 24, 48, 72 hpf).

Lethal endpoints were recorded at 24 hrs for the coagulation of embryos, lack of somite formation, and non-detachment of the tail. Non-development of eyes and lack of heartbeat were recorded at 48 hrs. Any positive outcome in these observations means that the zebrafish embryo is dead. Additionally, sublethal teratogenic endpoints were recorded including yolk sac edema, pericardial edema, lack of pigmentation, spinal deformity, and delayed hatching. Observations were recorded every 24 hrs until the end of the test at 72 hrs after the start of exposure.

For the evaluation of head and tail length, each well-plate containing 72 hrs embryos were submerged in an ice-cold container. After an hour, the dead embryos were harvested and preserved using 4% neutral formalin buffer solution, dechorionated and were mounted in glass slides. Embryos were then oriented, and a drop of the concentration was added to prevent the preparation from drying out. The preserved larvae were then measured (head and tail length) at 100× magnification using an *Olympus*™ CH10 Biological Microscope equipped with an ocular micrometer. A binomial convention was utilized and entered in a Microsoft Excel Starter 2010 template wherein each embryo/larva exposed would have a corresponding score for its overall effect. Normal zebrafish embryos/larvae were given a score of 0, while a score of 1 at each specified time points was given to embryos that exhibited lethal endpoints and those that were considered dead.

Data processing and analysis

Using IBM® SPSS® ver. 23 statistical software, Shapiro-Wilk test was used to obtain the normality of the mean ratios of lethal and sublethal endpoint occurrence, and mean values of head and tail lengths (µm) acquired from zebrafish groups exposed to ISO water (negative), 5% EtOH (positive), solvent (1% DMSO) and treatment groups. Homogeneity of variances was also determined through Levene's test on the same pool of data. Statistical significance of mortality, teratogenicity and/or changes in head and tail lengths arising as an effect of the metformin or glibenclamide treatment was analyzed using a pair-wise comparison under Kruskal-Wallis independent samples test between acquired data from ISO water, 5% EtOH, 1% DMSO, and treatment groups at a significance level of α=0.05. To compare the statistical significance between groups of metformin and glibenclamide concentrations, the Mann-Whitney U Test was used.

Ethical considerations

The study was submitted for review and approval to the Institutional Animal Care and Use Committee (IACUC) of the National Institutes of Health (NIH), University of the Philippines Manila.

Results

Validity of tests and output

Parameters indicated in the ZFET Standard Operation Procedure [21] were met and maintained throughout all the tests. The temperature level of all controls was constant at 26 ± 1°C, whereas the pH levels of reconstituted water ranged from 7.0-7.2. Additionally, electrical conductivity of the reconstituted water solution was consistent at around 600 ± 5 µS/cm.

Results revealed that all zebrafish embryos exposed to reconstituted/ISO water (negative control) underwent normal development at 24 hpf, 48 hpf, and exhibited 100% hatching rate at 72 hpf, showing 100% vitality. Conversely, zebrafish embryos exposed to 5% EtOH (positive control) revealed 100% mortality as all embryos exhibited absence of heartbeat at 48 hpf. Results further revealed significant differences between the percent vitality of positive and negative controls ($\chi 2$ =294.98, *df*=14, *n*=450, *p<0.001*). The possibility of the 1% DMSO solvent for metformin and glibenclamide, as a confounding variable was eliminated based on the observation that less than 3% of the embryos exposed to 1% DMSO exhibited coagulation, while normal development was observed for the rest of the zebrafish embryos in the solvent control.

The test for head and tail length measurements was validated using a pairwise comparison of the controls: reconstituted water (µhead=41.4 µm, µtail=202.4 ± 1.1 µm, *n=30*), 1% DMSO (µhead=41.3 ± 0.1 µm, µtail=202.7± 1.4 µm, *n=30*), and 5% EtOH (µhead=25.0 ± 2.6 µm, µtail=76.5 ± 2.9 µm, *n=30*). Results revealed no significant difference between the measured head (*t*=1.00, *df*=29, *p>0.05*) and tail lengths (*t*=0.39, *df*=58, *p>0.05*) of reconstituted water with 1% DMSO, but with a significant difference between the measured head (*t*=35.3, *df*=29, *p<0.001*) and tail lengths (*t*=37.2, *df*=37, *p<0.001*) of reconstituted water with 5% EtOH (µhead=25.0 ± 2.6 µm, µtail=76.5 ± 2.9 µm, *n=30*).

Lethal endpoints (24 and 48 hpf)

The presence of morphological abnormalities such as coagulation, non-formation of somites, and non-detachment of tail at 24 hpf as well as absence of heartbeat and non-development of eyes at 48 hpf are indicators of lethal endpoints during zebrafish embryonic development (Fig. 1).

These lethal endpoints are considered fatal as these deformities prevent further survival of the embryo until it is ready to hatch from its egg. Lethal endpoints were observed at 100 µg/L and 500 µg/L, in the metformin treatment, whereas 10 µg/L, 500 µg/L and concentrations from 500 mg/L to 2000 mg/L, exhibited lethal endpoints in the glibenclamide treatment (Fig. 2). Conversely, coagulation and absence of heartbeat were the lethal endpoints observed in glibenclamide treatment at concentration levels of 10 µg/L, 500 µg/L, 500 mg/L, 1000 mg/L and 2000 mg/L. However, to provide enough evidences that metformin and glibenclamide are lethal at these concentrations, a confirmatory statistical analysis using Kruskal-Wallis independent samples test and pairwise comparison against reconstituted water was done. Results revealed that only the concentration levels of 500 mg/L ($p<0.05$) and 2000 mg/L ($p<0.05$) glibenclamide were embryotoxic and lethal by effectively causing coagulation to exposed embryos.

Sublethal endpoints (72 hpf)

The presence of morphological abnormalities upon hatching or at larval stages at 72 hpf such as yolk sac edema, pericardial edema, and spinal deformity are considered sublethal endpoints. Evaluation of these deformities may indicate that the larvae may survive but does not guarantee maximum longevity and normal development into a healthy adult zebrafish. There were no observed cases of spinal deformity, pericardial and yolk sac edema in metformin treatments. Manifestation of pericardial edema was evident at 20 µg/L, 100 µg/L, 2000 µg/L, and 2000 mg/L glibenclamide concentrations. However, only at the level of 2000 mg/L was statistical difference observed from reconstituted water ($\chi 2$=25.9, df=11, n=360, $p<0.05$). Yolk sac edema cases were observed at 100 µg/L, 500 µg/L, 1000 µg/L, 2000 µg/L, 500 mg/L, 1000 mg/L, and 2000 mg/L glibenclamide concentrations. However, only at levels of 500 µg/L, 1000 mg/L and 2000 mg/L showed statistically different from reconstituted water ($\chi 2$=55.2, df=11, n=360, $p<0.001$) (Fig. 3). Spinal deformities were also observed in high concentrations of glibenclamide, particularly 500 mg/L, 1000 mg/L, and 2000 mg/L. However, statistical difference was only evident at 1000 mg/L and 2000 mg/L ($\chi 2$=48.6, df=11, n=360, $p<0.001$) (Fig. 3). Several sublethal endpoints were also observed simultaneously in a single embryo especially in higher concentrations of glibenclamide (Fig. 4).

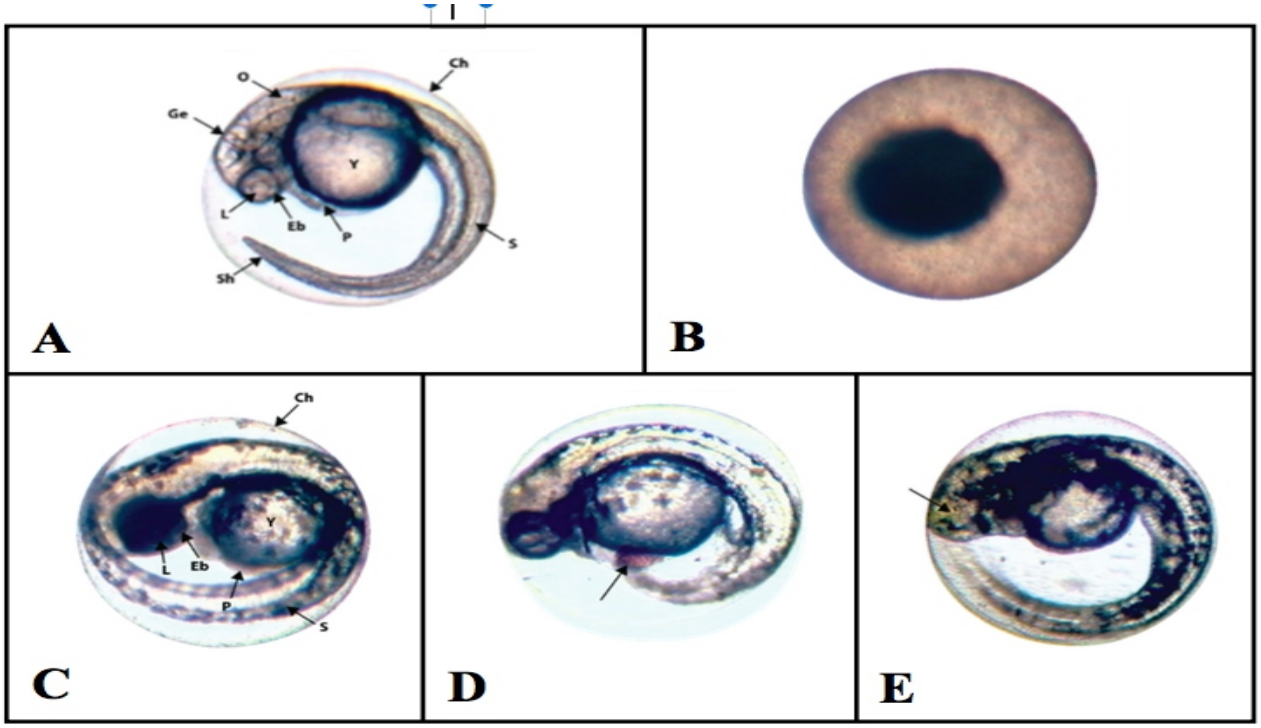

Figure 1. ***(A)*** *Normal embryo (24 hpf) with well-developed somites and distinct head and detached tail region; chorion (Ch); ear bud (O); brain (Ge); lens (L); eye buds (Eb); pericardium (P) yolk (Y); somites (S); tail (Sh); Embryo (24 hpf) exhibiting lethal endpoints: coagulation,* ***(B)*** *as seen in an embryo exposed to 10 µg/L of glibenclamide; 48 hours post fertilization developing control embryo,* ***(C)****; Embryo (48 hpf) exhibiting lethal endpoints: absence of heartbeat indicated by non-convulsion of heart* ***(D: →)*** *as seen in an embryo exposed to 500 µg/L of glibenclamide; and the zebrafish embryo exposed to 500 µg/L of metformin depicted in* ***(E: →)*** *shows non-development of eyes.*

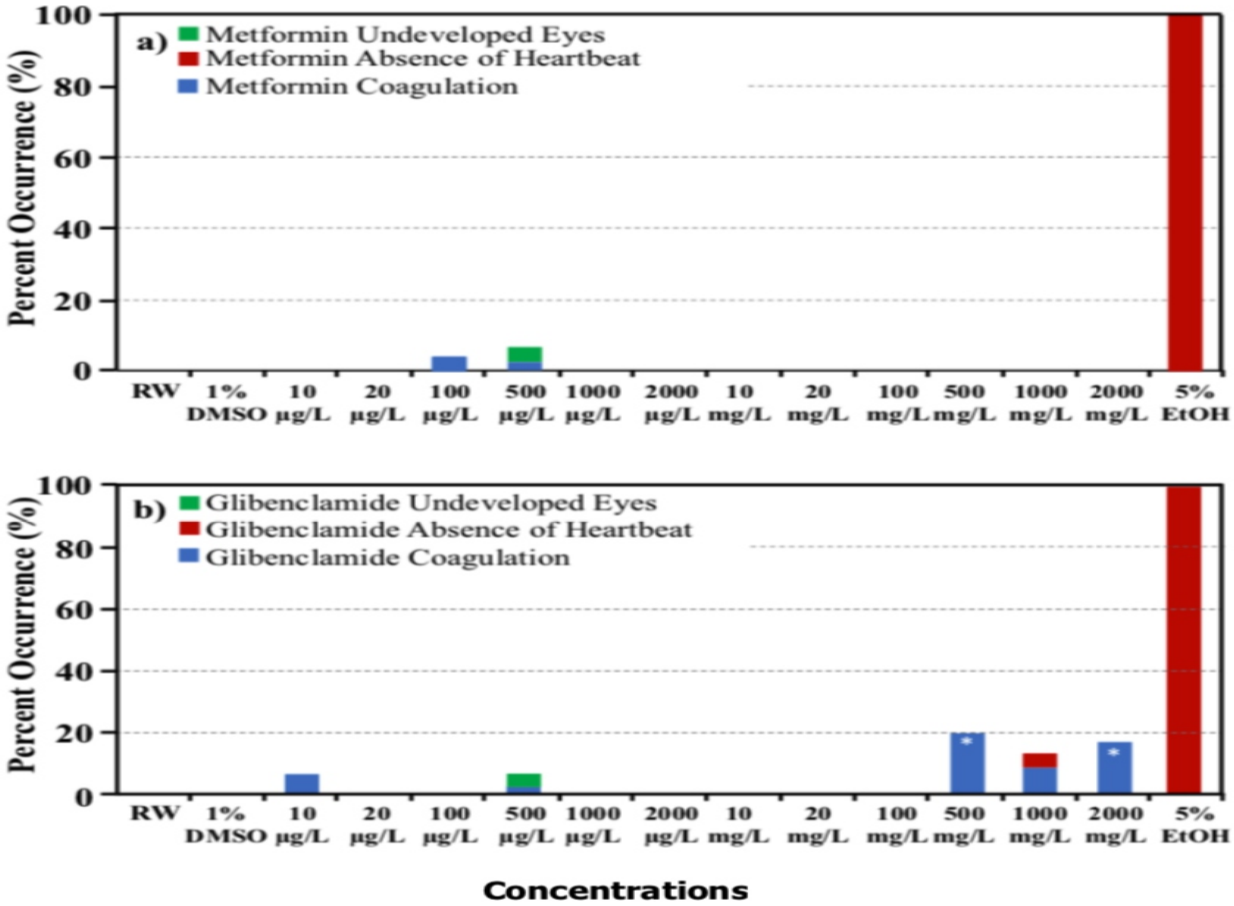

Figure 2. *Percent occurrence of zebrafish embryos exhibiting lethal endpoints: coagulation (CGN); non-formation of somites (NFS); non-detachment of tail (NDT); non-development of eyes (NDE) and absence of heartbeat (AOH) exposed to the different treatments of both metformin (M) and glibenclamide (G) at 24 and 48 hpf. Only glibenclamide treatment at 500 mg/L and 2000 mg/L showed significance against the control ($p<0.05$). 100% lethality in the positive control (5% EtOH).*

Advanced hatching (48 hpf) observed in metformin treatment group

Although not considered as a lethal nor a sublethal endpoint, an increasing trend in advanced hatching was observed with increasing metformin concentrations (Fig. 5).

Head and tail length

Measured head length in metformin treatment at 500 µg/L and treatments exceeding 2000 µg/L was significantly lower than the mean head length of the reconstituted water ($\chi 2$=362.9, df=14, n=360, $p<0.05$). These results suggest that at these concentrations, metformin can cause microcephaly in exposed embryos. On the other hand, measured values of head length in glibenclamide revealed no significant difference with the mean head length of the reconstituted water ($\chi 2$=271.6, df=14, n=360, $p>0.05$) (Fig. 6a). The mean tail length of metformin at concentrations covering 100 µg/L to 2000 mg/L were significantly ($p<0.05$) lower than the mean head length of reconstituted water. On the other hand, the mean tail length of glibenclamide at concentration levels from 10 mg/L to 2000 mg/L was significantly higher than the mean tail length of reconstituted water ($\chi 2$=367.67, df=14, n=450, $p<0.001$) (Fig. 6b).

Comparative assessment of metformin and glibenclamide

There were no significant ($p>0.05$) differences in the percent mortality between metformin and glibenclamide treatments. However, a comparison between the two treatments on percent occurrence of sublethal endpoints shows that embryos exposed to glibenclamide treatment has a statistically higher percent teratogenicity ($p<0.05$) since metformin has been previously observed to be non-teratogenic. For the mean head lengths, the two treatments were compared across concentrations and results revealed that the mean head lengths of the zebrafish embryo in glibenclamide treatment ranging from 500 µg/L to 2000 mg/L was significantly ($p<0.05$) higher than the metformin treatment. Figure 6a shows the decreasing head length trend

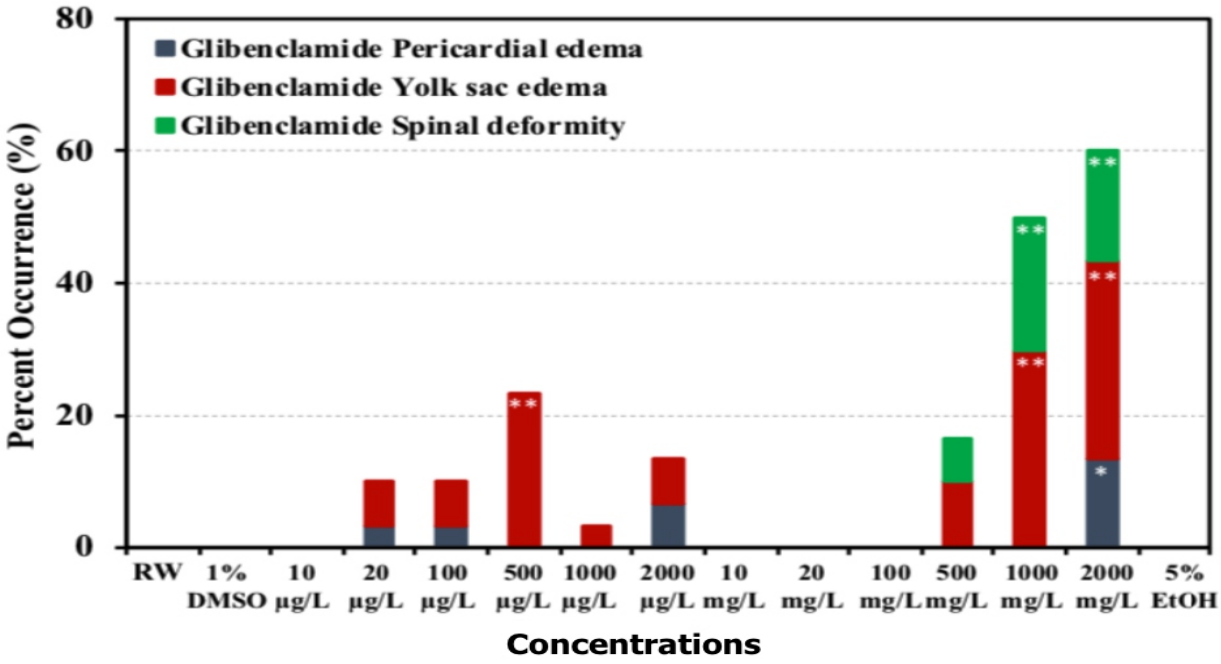

Figure 3. *Percent occurrence of zebrafish embryos exhibiting sublethal endpoints: spinal deformity (SD); yolk sac edema (YSE); and pericardial edema (PE) exposed to the different treatments of both metformin (M) and glibenclamide (G) at 72 hours post fertilization. No observed sublethal endpoints in reconstituted water (RW) and metformin while no data were shown for 5% EtOH since all embryos were considered dead already. [Statistical significance relative to control * (p<0.05) ** (P<0.001)].*

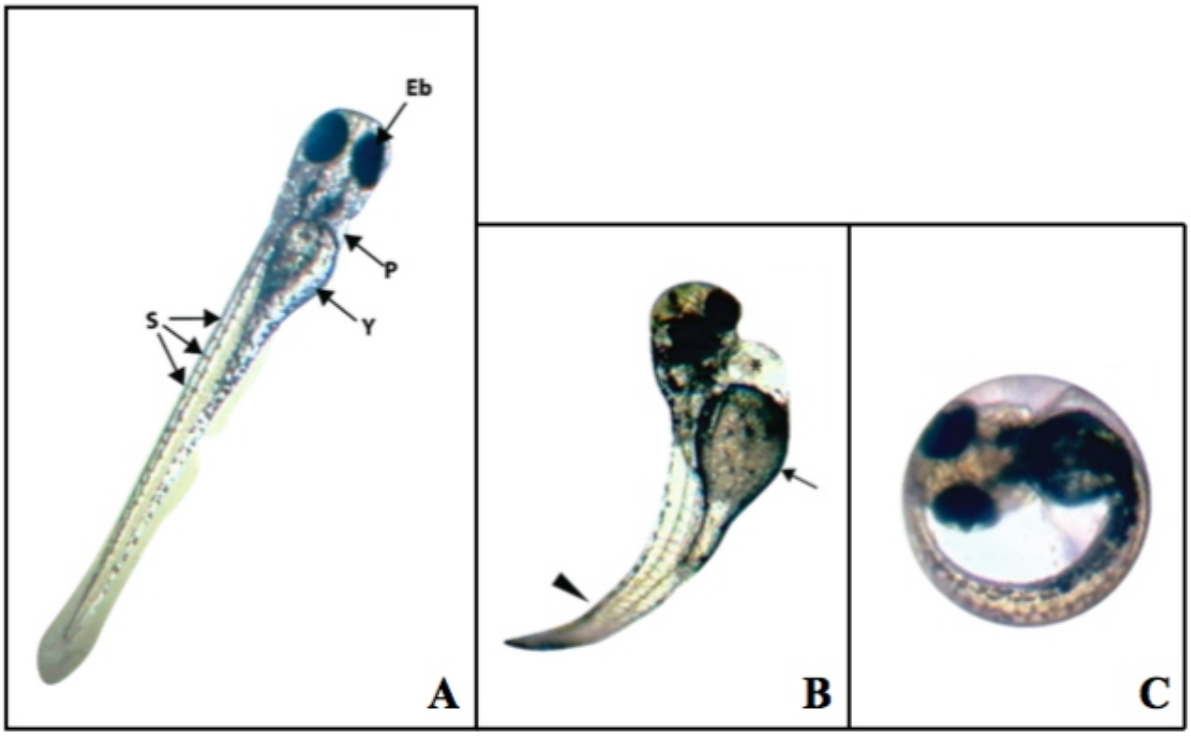

Figure 4. *72 hpf developing control embryo **(A)** eyeball (Eb), pericardium (P), yolk sac (Y) and spinal column (s); **(B-C)** showing embryos manifesting sublethal endpoints: pericardial edema (B: *) as seen in an embryo exposed to 2000 mg/L of glibenclamide as well as yolk sac edema (B:→) and spinal deformity (arrowhead); and the embryo exposed to 100 ug/L of glibenclamide depicted in **(C)** shows delayed hatching even until 72 hpf.*

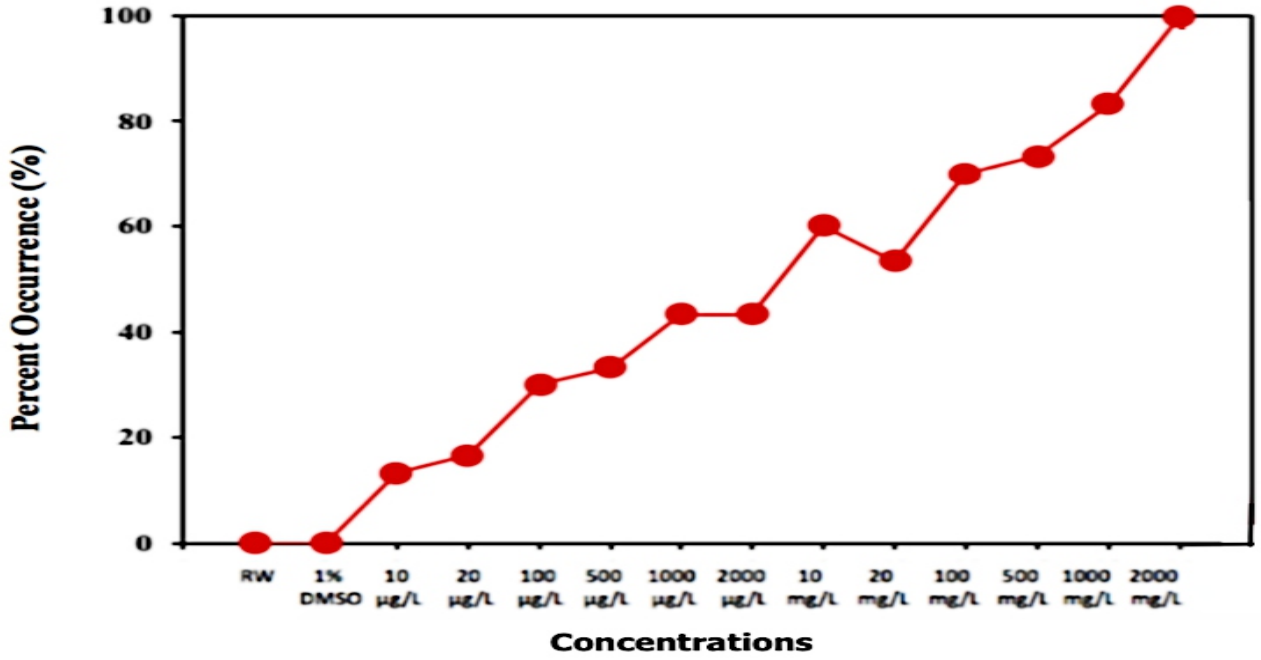

Figure 5. *Advanced hatching observed in zebrafish embryos exposed to metformin concentrations relative to the control (RW).*

of zebrafish embryos for metformin and the consistency of head lengths for glibenclamide at varying levels of concentration. To compare mean tail lengths, zebrafish embryos with glibenclamide treatment were observed to have an increasing tail length that was significantly ($p<0.001$) higher in all the treatment concentrations compared to that of the metformin. Figure 6b shows the decreasing tail length of the zebrafish embryos in the metformin and increasing tail length of glibenclamide across varying concentrations.

Discussion

Metformin

Several studies reported that metformin crosses the placenta freely and exposes the fetus to therapeutic concentrations [23-29]. These results suggest that the use of metformin during pregnancy raises concern regarding its potential adverse effects on the fetus. However, case studies involving women with T2DM and GDM are more complex, since birth defects are increased by maternal diabetes itself and is often difficult to distinguish whether a malformation is a consequence of the disease or the pharmacologic therapy [30].

In this study, well-plate metformin exposure in zebrafish embryos at dose range found in umbilical vein (*i.e.*, 10 µg/L-1000 µg/L) and dose range of maternal intake (*i.e.*, 10 mg/L-2000 mg/L) (*e.g.*, [24]) was carried out to observe embryotoxic and teratogenic potentials, which is independent of the risk factors provided by T2DM and GDM. The lethal endpoints observed at both low and high dose ranges pose no significant risk for the developing embryos. Findings are consistent with the retrospective human cohort studies, which also reported no significant lethality among the infants of women who conceived while taking metformin [3,31]. Furthermore, in animal studies on metformin, Tuchmann-Duplessis and Mercier-Parot [32] observed that the administration of 10 to 20-fold of the maximum human therapeutic metformin dose was not associated with a significant increase of malformations in pregnant rats. Similarly, Denno and Sadler [15] reported no significant risk in exposed mouse embryos, except a delay in neural tube closure observed at concentrations like those achieved in plasma in humans on therapy.

Conversely, Tartarin *et al.* [33] recently observed a significant risk in the reduction of testicular size in fetal and neonatal testes of mice prenatally exposed to metformin. Their results are important to consider in this study in lieu of the significant decrease in head and tail length of zebrafish embryo exposed to metformin, which may result from advanced hatching as embryos are not yet fully grown upon hatching. Metformin sensitizes cells to insulin by acting on metabolic pathways to promote catabolism and glucose uptake. Insulin is a peptide hormone and is known to

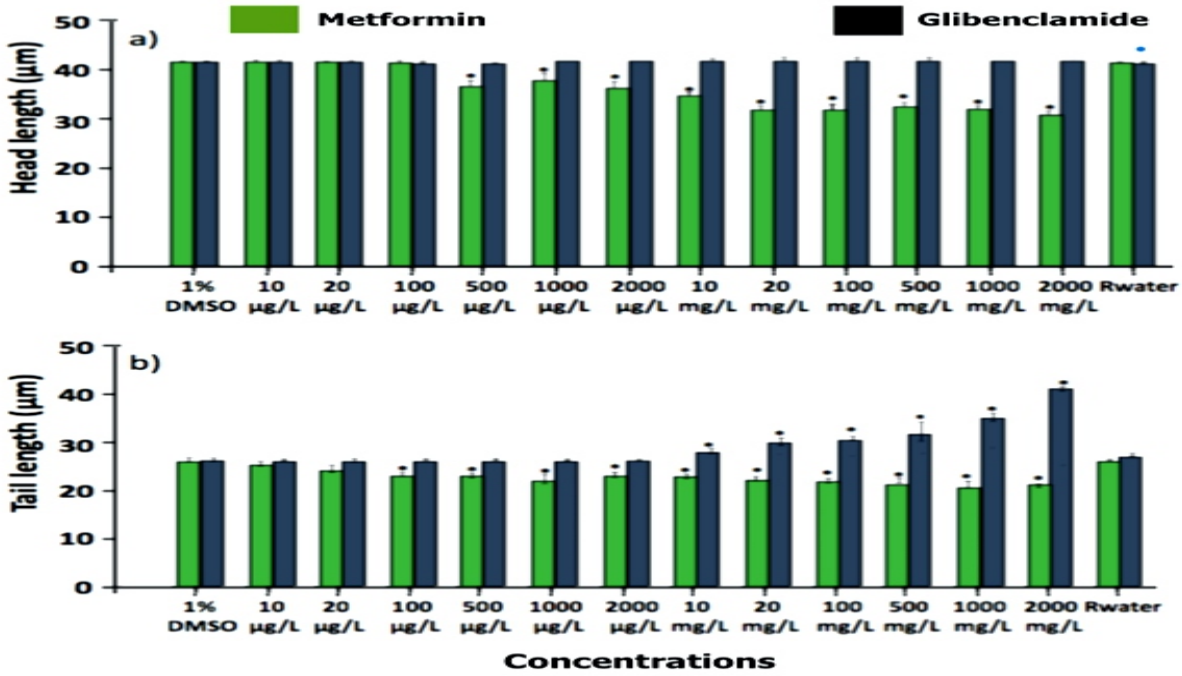

Figure 6. (a) *Head and* ***(b)*** *Tail length of zebrafish embryos exposed to various concentrations of metformin and glibenclamide for 72 h. Column RWater is the reconstituted water which served as the negative control while column 1% DMSO is the solvent control for both metformin and glibenclamide treatment groups.*

influence many aspects of endocrine signaling by affecting steroid synthesis [34]. The link between insulin signaling and steroidogenesis indicates the potential for antidiabetic drugs including metformin to act as endocrine disruptors. The enzymes involved in the steroid biosynthesis pathway are being recognized as important targets for the actions of various endocrine-disrupting chemicals. Interferences with steroid biosynthesis may result in impaired reproduction, alterations in (sexual) differentiation, growth, and development of certain cancers [35].

Expression of vitellogenin (VTG) mRNA has been demonstrated as a sensitive marker of endocrine disruption in zebrafish [36] and in North American fathead minnow [34] reported that metformin causes induction of VTG expression in male fathead minnow liver despite the concentration of metformin was limited to 40 µg/L. It follows that the significant up-regulation of VTG may be observed in higher concentrations of metformin exposure as an indicative of the potential for greater endocrine-disrupting impacts. Therefore, a decreasing trend in head and tail length of zebrafish embryo observed at significant concentrations, which are higher than 40 µg/L metformin (500 µg/L and >2000 µg/L for head length and >100 µg/L of metformin for tail length) may be related also to an increase in the expression of VTG mRNA causing endocrine-disrupting effects.

In this study, the incidence of advanced hatching was observed to be significantly ($p<0.05$) greater at high concentrations of metformin. The results are consistent with the results of a randomized comparative study by Rowan *et al.* [37] on fetal outcomes of pregnant women treated with metformin and insulin where the rates of spontaneous and iatrogenic preterm births were higher in women treated with metformin than those with insulin. Despite having observed a 100% occurrence of advanced hatching of embryos, the highest concentration of metformin conceived was at 0% teratogencity. While there are several studies [37-39] on metformin's protective effect on mothers by decreasing the frequency of risk factors posed by T2DM and GDM, there are no studies that show its protective effect on embryos in the aspect of lowering the risk of congenital malformations in embryos. Although statistically ($p>0.05$) insignificant in the study, the chance that its association is discarded could be the subject for future investigations.

Glibenclamide

Comparing the transplacental transfer of four sulfonylurea derivatives via *in vitro* perfusion of human placental cotyledons, Elliot *et al.* [40] reported that glibenclamide transport was only to a very small extent relative to chlorpropamide, tolbutamide, and glibizide. These results

suggest no danger as advertised on glibenclamide. However, Sivan *et al.* [41] examined the placental transport of glibenclamide *in vivo* in pregnant rats via radio labelling. The levels of injected tritriated glibenclamide, C^{14} albumin (negative control), and C^{14} diazepam (positive control) were measured and compared in both maternal and fetal tissue. Their results revealed that the concentrations of radioactive glyburide in fetal tissue is statistically equal to its concentrations in maternal blood and to diazepam concentrations in fetal tissue, which is known to cross readily through the placenta. Their findings suggest that glibenclamide crosses the placenta of pregnant rats in significant amounts and should therefore be considered with caution as a hypoglycemic agent in the treatment of GDM.

In this study, well-plate glibenclamide exposure to zebrafish embryos at the dose range found in umbilical vein and maternal tissues was conducted to compare with metformin in terms of embryotoxic and teratogenic activity [12]. Results showed that glibenclamide induces both embryotoxic (lethal) effects, specifically coagulation as well as teratogenic (sublethal) effects, which include delayed hatching, pericardial edema, yolk sac edema, and spinal deformities, particularly at high doses. On the other hand, for low glibenclamide doses, the teratogenic effect observed were delayed hatching and yolk sac edema. Additionally, in contrast to metformin, no significant ($p>0.05$) differences were observed in the risk of micro- or macrocephaly. However, a significant ($p<0.05$) increase in the mean tail length of zebrafish larvae exposed to 10 mg/L to 2000 mg/L glibenclamide concentrations was observed.

Previous studies have reported that exposure of embryos to high glibenclamide doses led to overstimulation of fetal B-cells, resulting in severe hyperinsulinemia in the embryo [42,43]. Also, glibenclamide induces fetal hyperinsulinemia and, consequently, increased plasma triglycerides and increased transport and storage of fatty acids into the embryo [44,45]. In effect, the body morphologically changes in response to the import of these molecules and causes macrosomia, or occurrence of large-size or heavier fetus [45]. Macrosomia could be the same case for glibenclamide exposed embryos of the study, where a significant ($p<0.05$) increase in the tail length of zebrafish embryo was observed in higher concentrations of glibenclamide, but no significant ($p>0.05$) difference in head length.

Several studies have shown that glibenclamide exposed neonates are also significantly hypoglycemic apart from the previously observed neonatal macrosomia. For example, Mukhopadhyay *et al.* [46] reported that 30 randomized patients with GDM were given glibenclamide and another 30 were given insulin to compare their neonatal and perinatal outcomes. Their study revealed that neonatal hypoglycemia and macrosomia were simultaneously observed to be significantly higher in infants whose mother was treated with glibenclamide compared to insulin and this result is consistent with other studies. Balsells *et al.* [47] compared glibenclamide with insulin and concluded that glibenclamide treatment for gestational diabetes is associated with simultaneous macrosomia and neonatal hypoglycemia. Odiba *et al.* [48] also reported that treatment of gestational diabetes with glibenclamide is associated with 1.4 times increased risks of neonatal hypoglycemia and macrosomia.

Exposure of glibenclamide in zebrafish embryos also induced spinal deformities [49]. Previous studies [50,51] induced the condition by fasting pregnant mice for short durations during early gestation, which resulted in numerous skeletal abnormalities that were highly associated in mouse embryos undergoing tricarboxylic-acid (TCA) cycle stress in response to the inherently low carbohydrate source [52].

Conclusion

This study shows that metformin is a more favorable alternative to insulin than glibenclamide considering that the latter induces significant lethal and teratogenic effects, including coagulation, pericardial edema, yolk sac edema, spinal deformities and a significant increase in tail length of exposed zebrafish embryos, in doses known to constitute fetal and maternal serum. However, the potential of metformin to effectively induce microcephaly was observed in significantly decreasing head lengths of exposed zebrafish embryos. Further studies are needed on the passage of the drug (metformin and/or glibenclamide) to the chorionic barrier of zebrafish embryos and to relate it to the lethality and morphological endpoints that were observed in the embryos. Should it pass through the chorionic barrier of zebrafish embryos, it may be crucial to consider at which specific locations these drugs accumulate.

Competing Interest Statement

The authors declare that they have no significant competing financial, professional, or personal interests that might have influenced the presentation of the work described in this paper.

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