EMBRYO BIOLOGY

Antidiabetic drug metformin affects the developmental competence of cleavage-stage embryos

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Abstract

Purpose Metformin is the most commonly prescribed drug in the management of metabolic disorders such as polycystic ovarian syndrome (PCOS) and gestational diabetes in women of reproductive age. Insulin-sensitizing effect of metformin helps in improving from PCOS features such as hyperandrogenism, anovulation, and infertility. However, its ability to cross placental barrier raises concern about safety of the drug on early embryonic development. In this study, we evaluated the effect of metformin on the ovarian function and embryo development.

Methods Adult Swiss albino female mice were administered with metformin (0, 50, 100, and 200 mg/kg body weight) for 4 weeks and assessed for reproductive function and preimplantation embryo development. Further, effect of metformin (0, 10, 25, 50, 100, 250, and 500 μg/mL) exposure to 2-cell-stage embryos was tested under in vitro conditions.

Results Metformin did not alter the body weight, blood glucose, ovarian weight, and follicular reserve. However, the early embryo development was significantly affected in mice treated with metformin in vivo at highest dose. Moreover, embryos which were exposed to metformin in vitro showed dose-dependent decline in blastocyst rate and hatching rate. Furthermore, at highest concentration of metformin (500 μg/mL), all the embryos were arrested at compaction stage.

Conclusion The study revealed that metformin affects the early embryonic development and raises concern about its use during conception.

Keywords Metformin · Polycystic ovarian syndrome · Blastocyst rate · In vitro maturation

Introduction

Metformin (dimethylbiguanide) is the most common and effective drug used in the treatment of type II diabetes mellitus and its associated metabolic complications. It exerts anti-hyperglycemic effect by decreasing intestinal absorption and hepatic production of glucose as well as increasing the peripheral glucose uptake [1]. Metformin is known to inhibit electron transport chain complex I (ETC I) and thereby decrease cellular adenosine tri-phosphate (ATP) level [2, 3]. The decreased ATP level in turn activates adenosine monophosphate (AMP)–activated protein kinase (AMPK), a key energy sensor, which triggers multiple signaling cascades to maintain energy homeostasis [4].

Owing to the vital role of metformin in metabolic and endocrine modulations, currently it is the best drug of choice for treatment of polycystic ovarian syndrome (PCOS), the condition which is observed in 10–15% females of reproductive age group [5]. PCOS is characterized by hyperandrogenism, insulin resistance, and obesity which results in anovulation and formation of cystic ovaries. Metformin inhibits steroidogenesis and androgen production by decreasing ATP and nicotinamide adenine dinucleotide (NAD^+) levels [6, 7].

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Metformin treatment regimen usually lasts for long duration in women of reproductive age group in order to minimize obstetric and pregnancy complications associated with PCOS. Studies have shown that metformin lowers pregnancy loss, reduces gestational diabetes and risk of birth defects [8–10]. On the contrary, Cochrane database showed that metformin treatment before and during assisted reproductive technology (ART) did not improve the live birth rate [11]. Further, a recent randomized double-blind controlled trial reported that metformin administration does not improve in vitro fertilization (IVF) outcomes in terms of live birth, fertilization, and implantation rate and showed similar multiple pregnancy and miscarriage rate compared with placebo [12].

It is well known that ATP is very essential for the resumption of meiotic events in oocytes [13], and therefore, reduction of ATP induced by metformin is speculated to interfere with oocyte and embryo development. It has been observed that metformin exposure causes arrest of bovine and porcine oocytes in germinal vesicle (GV) stage during in vitro maturation [14, 15]. Further, in vitro exposure of 2-cell-stage embryos to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) has shown to affect the blastocyst formation by decreasing the expression of tight junction proteins and trophectoderm differentiation which is associated with activation of AMPK [16]. Similarly, metformin-mediated AMPK activation has led to the reduction in expression of pluripotency genes such as octamer-binding transcription factor 4 (OCT4) and reduced expression protein 1 (REX1) in the blastocyst when 2-cell-stage embryos were exposed [17] suggesting its possible adverse effects on early embryo development. However, it is not clear whether metformin affects the ovarian function and whether maternal exposure to metformin has any detrimental effects on early embryos. The present study was designed to understand the consequences of metformin administration on ovarian reserve and pre- and post-implantation embryo development using a mouse model.

Materials and methods

Animal treatment

Healthy Swiss albino mice (8 to 10 weeks) maintained in Central Animal Facility, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal, were used in the study. The mice were maintained under standard conditions of temperature (25 ± 2 °C), humidity (45–55%), light (12:12 h of light and dark), and food and water ad libitum. Animal handling and experimental procedures were done according to the institutional guidelines and the study protocols were approved by the Institutional Animal Ethics Committee of Kasturba Medical College (IAEC/KMC/25/2014). Metformin (1,1-dimethylbiguanide hydrochloride, Cat. No. D150959, Sigma Aldrich, USA)

was dissolved in normal saline (0.9% NaCl) and injected at a dose of 50, 100, and 200 mg/kg body weight for 4 weeks, daily by intraperitoneal (i.p.) route. The doses of metformin used in this study are clinically relevant [18] and are based on previous studies [19, 20]. Control group mice received normal saline. The animals were monitored for body weight changes every week during the course of metformin administration. After the completion of treatment, the mice were sacrificed and assessed for various parameters (Fig. 1). A minimum of 6 animals were used for each independent experiment.

Estimation of blood glucose

After the completion of metformin treatment, random blood glucose was analyzed in the morning (between 9 and 11 am) using a glucometer (Roche Diagnostics, Germany) by placing a drop of blood obtained by cutting the tail tip over the strips attached to the glucometer. Glucose concentration was expressed as milligrams per deciliter.

Estimation of serum testosterone and estradiol level

The blood was collected by puncturing the heart and incubated at room temperature for 2 h. The serum was separated by centrifuging the blood at 2000 rpm for 10 min at room temperature and stored at -80 °C until further analysis. Enzyme-linked immunosorbent assay (ELISA) was performed using specific kits to assess the concentration of testosterone (Cat. No. DE1559, Demeditec Diagnostics Gmbh, Germany) and estradiol (Cat. No. DEH3355, Demeditec Diagnostics Gmbh, Germany). Briefly, standard solutions and serum (25 μL) were mixed with enzyme conjugate solution (200 μL) in 96-well plates and incubated for 1 h at room temperature. The contents were removed and washed 3 times with washing solution. The substrate solution (200 μL) was added and incubated for 15 min at room temperature. The reaction was stopped by adding stop solution (100 μL) and absorbance was read at 450 nm using an ELISA plate reader (LISA Plus, Rapid Diagnostics, India). The concentrations of hormones in serum were calculated by standard curve obtained. The ELISA kits used for the study were able to detect testosterone and estradiol in the range as low as 0.083 ng/mL and 3.94 pg/mL respectively. The antibodies coated in wells were having 100% specificity for respective hormones with negligible non-specific interaction to other steroids.

Follicle count

To understand the effect of metformin on ovarian reserve, the number of follicles was assessed by the method described earlier [21]. Briefly, one ovary from each mouse was subjected to enzymatic digestion by incubating in dissociation medium containing collagenase type IV (5 mg/mL, Cat. No. 17104019, Invitrogen, USA), trypsin (1 mg/mL, Cat. No. RM173,
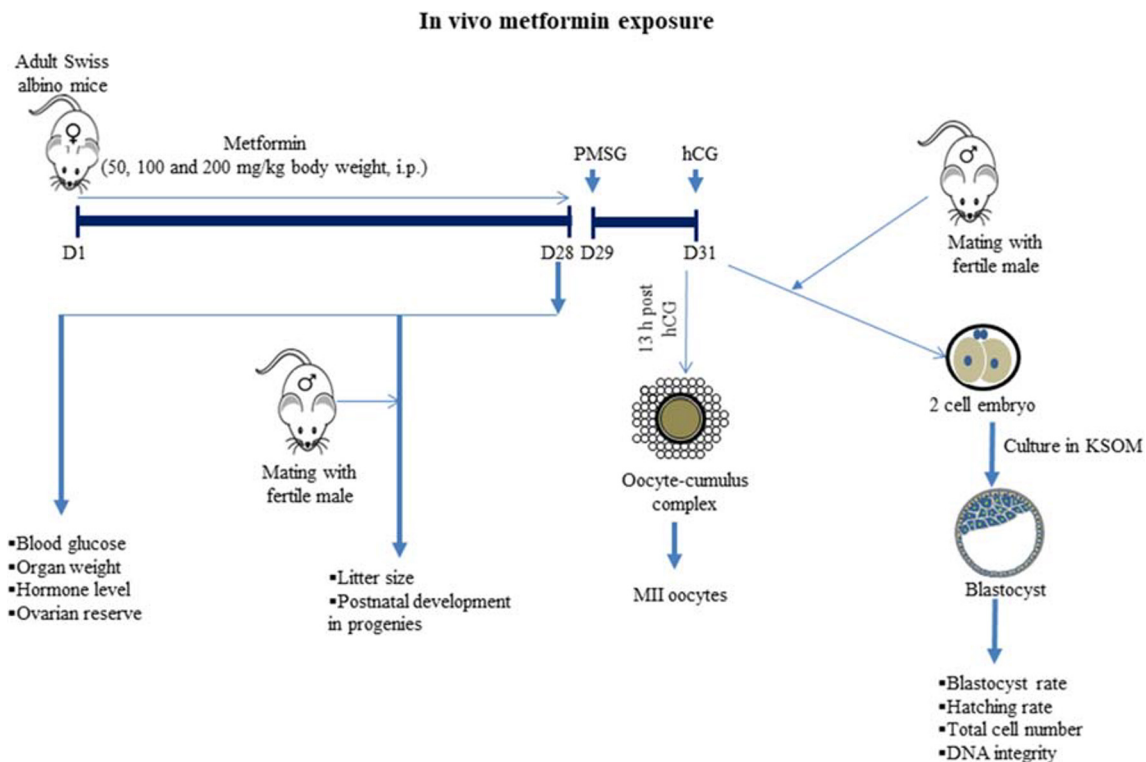
Fig. 1 The study outline to understand the effect of in vivo exposure of metformin on female reproductive system, ovarian reserve, preimplantation embryo development, and transgenerational changes

Himedia, India), and hyaluronidase (0.1 mg/mL, Cat. No. H4272, Sigma Aldrich, USA) for 1 h at 37 °C and then dispersed by gentle mechanical agitation. The follicles were counted manually under an inverted microscope (1X70, Olympus, Tokyo) and categorized into primordial, primary, and secondary follicles.

Superovulation

To collect mature oocytes and 2 cell embryos, female mice were primed with 5 IU pregnant mare serum gonadotropin (PMSG, Cat. No. G4877, Sigma Aldrich, USA) 24 h post last metformin injection. At 48 h post-PMSG, 10 IU human chorionic gonadotropin (hCG, Ovutrig VHB Life Sciences Inc. GenBiotech, Mumbai, India) was injected to each mouse to induce ovulation [22].

Oocyte collection

At 12-13 h post-hCG, the oocyte cumulus complexes (OCCs) were collected in potassium-supplemented simplex optimized medium (KSOM), and the oocytes were denuded by incubating OCCs in KSOM media containing hyaluronidase (0.1 mg/mL, Cat. No. H4272, Sigma Aldrich, USA) for 1 min at 37 °C. The oocytes were segregated into GV, MI (metaphase I), MII (metaphase II), degenerated, and fragmented oocytes. The number of MII (mature) oocytes was recorded and expressed as MII oocytes retrieved per animal.

Embryo developmental potential

After the administration of hCG, superovulated female mice were mated overnight with 10–12-week-old proven fertile Swiss albino male mice. Mating was confirmed by the presence of vaginal plug on the next morning which is considered 0.5 days post-coitus (dpc). Females were humanely sacrificed by cervical dislocation at 1.5 dpc, and 2-cell-stage embryos were retrieved from oviducts in Earle's balanced salt solution (EBBS) medium containing 0.1% BSA. The embryos were cultured in KSOM media at 37 °C and 5% CO_2 till blastocyst stage. The number of 2-cell-stage embryos cultured and number of blastocyst obtained were used to calculate blastocyst rate and hatching rate.

Total cell number and apoptotic index in blastocyst

The total cell number and DNA integrity in the blastocyst were analyzed by terminal deoxyribonucleotidyl transferase–mediated dUTP (Deoxyuridine triphosphate) nick end labeling (TUNEL) assay as described earlier [21]. The blastocysts were fixed in 4% paraformaldehyde (PFA) and permeabilized in 0.5% Triton X-100 in 0.1% sodium citrate. Later, these blastocysts were incubated with TUNEL reaction mixture (Cat. No. 1215792910, Roche GmbH, Germany). Labeled blastocysts were counterstained with nuclear stain 4′6-diamidino-2-phenylindole (DAPI, Cat. No. D9542, Sigma Aldrich, USA) and mounted on a slide using fluorescence mounting medium (Cat No. S3023, DAKO,

USA). Total cell number and apoptotic nuclei (TMR red–positive nucleus) were scored for each blastocyst by observing under a fluorescent microscope (Imager-A1, Zeiss, Gottingen, Germany), and the apoptotic index (apoptotic cell/total cell number × 100) was calculated.

Fertility assessment of metformin-treated female mice and transgenerational changes

The metformin-treated female mice were mated with proven fertile males by housing them in a ratio of 1:1. The mating was confirmed by observing vaginal plug. The mating efficiency, pregnancy rate, and litter size were noted. The post-natal development was assessed by monitoring developmental milestones such as eye opening, fur development, body weight, and any other phenotypic anomalies.

Effect of metformin exposure on early embryo development: in vitro studies

A schematic representation of in vitro metformin treatment is depicted in Fig. 2. To understand the effect of metformin on the development of oocytes and embryos, these were cultured in medium containing different concentrations of metformin (0, 10, 25, 50, 100, 250, and 500 µg/mL). The concentrations were selected based on the earlier study [23].

GV oocyte collection and in vitro maturation

The ovaries were collected from mice in natural cycle (unstimulated ovary). The cortical region of the ovaries was gently teased using a fine needle to obtain germinal vesicle (GV) stage oocytes from the secondary/tertiary follicles. GV oocytes were further subjected to in vitro maturation by culturing in 20-µL droplets of Dulbecco's modified Eagle's medium (DMEM, Cat. No. D5648, Sigma Aldrich, USA) supplemented with 1% non-essential α-amino acids (Cat. No. M7145, Sigma Aldrich, USA), 1% insulin-transferrin-selenium (ITS, Cat. No. 51500–056, Gibco, USA), 0.05% pyruvate, and 0.3% bovine serum albumin (BSA). Oocytes were incubated at 37 °C in 5% CO_2 for 24 h and then assessed for nuclear maturity. Oocytes with one polar body (PB) in perivitelline space were considered as mature (MII) oocytes.

Estimation of reactive oxygen species in MII oocytes by 2′,7′-dichlorofluorescin diacetate staining

MII oocytes were incubated with 10 µM 2′,7′-dichlorofluorescin diacetate (DCFDA) (Cat. No. D6883, Sigma Aldrich, USA) in culture medium (DMEM) droplet for 30 min at 37 °C and 5% CO_2. The oocytes were washed 3 times with media and transferred to a clean microscopic slide immediately and visualized under a fluorescent
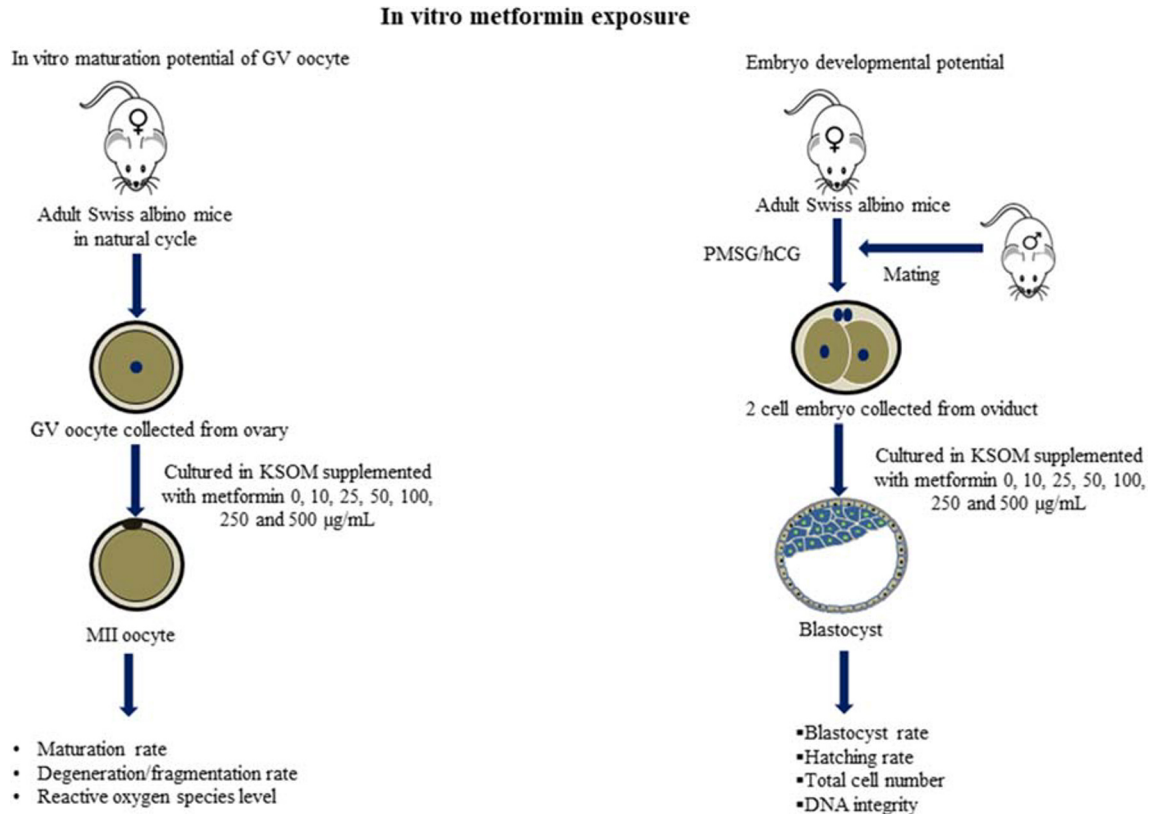

Fig. 2 The study outline to understand the effect of in vitro exposure of metformin on oocyte maturation and early embryo development

microscope (Imager-A1, Zeiss, Gottingen, Germany). The images were grabbed for all the oocytes on the same day to avoid variations in fluorescence intensity using Q imaging (Micropublisher 5.0 RTV, Canada) by keeping constant exposure time. ROS intensity was measured using Q-capture software (Q Capture Pro 7, USA). The intensity corresponds to the ROS level in the oocytes and expressed as lum units.

Developmental potential of embryos following metformin exposure in vitro

Healthy female mice were superovulated by administering 5 IU PMSG and 10 IU hCG after a gap of 48 h and mated with proven fertile healthy male mice (10–12 weeks) overnight. Mating was confirmed by observing vaginal plug. At 36 h post-hCG, 2-cell-stage embryos were collected from the oviduct of female mice. The embryos were washed in KSOM medium droplets (50 μL) and cultured in media containing different concentrations of metformin (0, 10, 25, 50, 100, 250, and 500 μg/mL) till 4.5 dpc at 37 °C and 5% CO_2. Embryos were scored at every 24 h interval by monitoring them under an inverted microscope (IX70, Olympus, Tokyo) fitted with a thermoregulated stage. The developmental potential of the embryos was assessed by calculating blastocyst rate and hatching rate.

Statistical analysis

All the data were presented as mean and standard error (mean ± SE) except for embryo and oocyte developmental parameters which were represented in percentage. The statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison for the data which obey Gaussian distributions and Kruskal-Wallis test for the data which did not obey Gaussian distributions using GraphPad InStat 3.0 statistical package (GraphPad Inc., version 3.06, USA). The percentage values were analyzed between the groups by contingency table followed by Fisher's exact test.

Results

Effect of metformin on body weight, organ weight, blood glucose level, and hormone level

Daily administration of metformin for 28 days did not alter the body weight in female mice at any of the doses injected. Mice from all the groups gained ~ 2 g at the end of injection period (4 weeks) at which stage animals were 12–14 weeks old. Blood glucose level, ovarian weight, and liver weight were not altered in any of the metformin-treated groups. However, a significant reduction was observed in weight of spleen

($P < 0.05$) in highest dose of metformin (200 mg/kg) compared with control. Interestingly, liver to spleen (L/S) ratio was increased in a dose-dependent manner where the highest dose group showed 1.7-fold increase compared with control. However, these changes were statistically non-significant. The level of reproductive hormones such as estradiol and testosterone were not altered by metformin treatment when compared with control (Table 1).

Effect of metformin on ovarian reserve

Metformin treatment did not affect follicular reserve as indicated by similar number of primordial, primary, and secondary follicles in the control and metformin group (Table 2). Similarly, the number of ovulated MII oocytes retrieved from superovulated mice was not altered by metformin administration at all three doses compared with control (Fig. 3).

Effect of in vitro exposure of metformin on oocyte maturation

The effect of metformin on the nuclear maturation of GV oocytes was assessed by subjecting them to in vitro maturation. Maturation rate in control oocytes was 55.7% which was not affected by metformin up to 500-μg/mL concentration. Significantly high percentage of oocyte degeneration was observed in 500 μg/mL concentration ($P < 0.01$) indicating that metformin at high dose has toxic effects on the oocytes (Fig. 4). At lower concentrations, metformin induced significant increase in the intracellular reactive oxygen species (ROS) level (10 to 50 μg/mL, $P < 0.001$, and 100 μg/mL, $P < 0.01$). At higher doses (250 and 500 μg/mL), there was no significant difference in the intracellular ROS level (Fig. 5).

Effect of metformin on preimplantation embryo development

The number of 2-cell-stage embryos retrieved from female mice administered with different doses of metformin was similar to that of the control group suggesting that metformin did not have any effect on ovulation and fertilization (Table 3). The incidence of fragmented oocytes was increased by 2-fold in the 200 mg/kg metformin group compared with control. On 3.5 dpc, the number of embryos that reached blastocyst stage was similar in the control and 50 mg/kg group. In the 100 mg/kg group, the blastocyst rate was non-significantly lower compared with control. However, a significant reduction ($P < 0.01$) in blastocyst rate was observed in the 200 mg/kg metformin group compared with control. On 4.5 dpc, the blastocyst rate was similar in the 50 and 100 mg/kg metformin group compared with control, while in the 200 mg/kg group, the embryos reaching blastocyst stage was significantly lower ($P < 0.0001$) compared with control

Table 1 The effect of various doses of metformin on body weight, organ weight, blood glucose, and hormone level in Swiss albino mice

Metformin dose (mg/kg)	Body weight gain (g) ($n = 36$)	Blood glucose (mg/dL) ($n = 8$)	Ovary weight (mg) ($n = 32$)	Spleen weight (mg) ($n = 6$)	Liver weight (g) ($n = 6$)	Liver/ spleen ratio	Estradiol (pg/ mL) ($n = 5$)	Testosterone (ng/mL) ($n = 5$)
0	2.59 ± 0.24	122.63 ± 7.81	9.54 ± 0.54	188.53 ± 18.70	1.31 ± 0.72	6.96	19.51 ± 3.52	0.34 ± 0.05
50	2.63 ± 0.37	120.25 ± 4.31	10.16 ± 0.59	154.15 ± 15.30	1.11 ± 0.59	7.18	18.85 ± 2.56	0.25 ± 0.03
100	2.39 ± 0.27	119.63 ± 6.58	10.10 ± 0.63	140.52 ± 19.25	1.12 ± 0.45	8.02	16.06 ± 3.11	0.22 ± 0.04
200	2.83 ± 0.37	131.88 ± 5.49	9.36 ± 0.30	105.98 ± 8.76[a]	1.26 ± 0.47	11.98	17.34 ± 3.65	0.20 ± 0.02

Data were expressed as mean ± SE. [a] $P < 0.05$ compared with the control group

with higher percentage of fragmentation or degeneration ($P < 0.0001$) rate. Similarly, the number of blastocysts hatched on 3.5 dpc was significantly lower in the 100 and 200 mg/kg group ($P < 0.01$). On 4.5 dpc, hatching rate was significantly lower only in 200 mg/kg ($P < 0.01$) which suggests slow development of embryos induced by metformin. In addition, the blastocysts from the 200 mg/kg group showed significantly lower total cell number ($P < 0.01$) than control. However, metformin treatment did not have any significant effect on the DNA integrity of the blastocysts.

Effect of in vitro exposure of metformin on the development potential of 2-cell-stage embryo

To confirm this observation made on effect of metformin on embryo development, we carried out an in vitro experiment where 2-cell-stage embryos (1.5 dpc) were exposed to various concentrations of metformin (10–500 μg/mL) and assessed for their developmental potential (Table 4). At 24 h later, there was no significant difference observed in metformin-treated embryos except in the highest concentration (500 μg/mL) in which there was a significant decrease in the embryos which progressed to 6–8 cell stage ($P < 0.01$). Similarly, at 48 h later (3.5 dpc), compaction rate was significantly lower in this concentration ($P < 0.0001$). In the rest of the concentration of metformin, even though the morula rate was not affected, reduction in blastocyst rate was observed. At and above 25 μg/mL concentration, the blastocyst rate decreased in a dose-dependent manner. However, a significant reduction ($P < 0.001$) was observed only in 250 and 500 μg/mL concentration. On 4.5 dpc, blastocyst rate was similar in lower

concentrations of metformin (10–100 μg/mL) compared with control. In 250 μg/mL, only 7.69% ($P < 0.0001$) of the 2-cell embryos reached blastocyst stage, and in the 500 μg/mL group, none of the embryos developed to blastocyst ($P < 0.0001$) compared with control. Hatching rate was significantly decreased in 100 μg/mL ($P < 0.01$), 250 μg/mL ($P < 0.001$), and 500 μg/mL (0%, $P < 0.001$) concentration compared with control. The analysis of total cell number in the blastocyst revealed that the total cell number was decreased in 50 μg/mL and 100 μg/mL metformin concentration compared with control. However, values are statistically significant only in the 100 μg/mL ($P < 0.01$) group. TUNEL assay in blastocyst showed that metformin exposure did not affect the DNA integrity of the blastocysts in any of the concentrations tested.

Transgenerational changes induced by maternal exposure to metformin

The fertility potential of female mice treated with metformin was assessed by mating with healthy fertile males and mating was confirmed by the presence of vaginal plug. Compared with control (86%), mating efficiency was not altered significantly in the 50 mg/kg (71%), 100 mg/kg (86%), and 200 mg/kg (100%) metformin-treated group. The litter size in metformin-treated mice was also similar to that of control. There was no significant difference in the post-natal development of litters born to metformin-treated mothers with respect to survival and other developmental milestones such as fur development and eye opening. The litters born to mothers treated with 200 mg/kg of metformin showed decrease in

Table 2 The effect of various doses of metformin on follicular reserve in Swiss albino mice ($n = 9$)

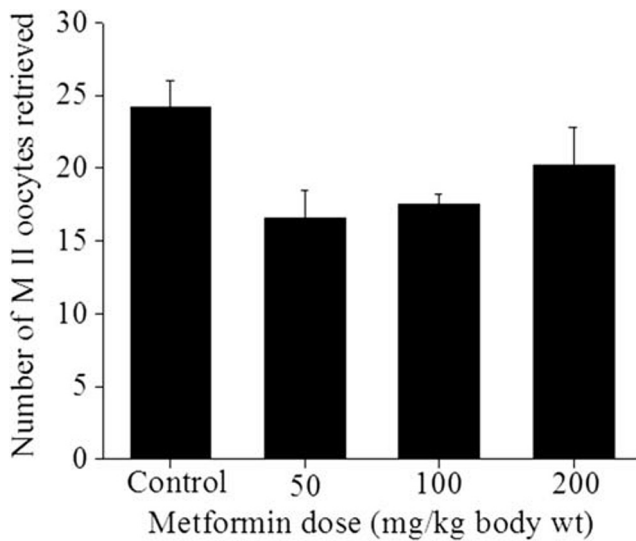| Metformin dose (mg/kg)	Total number of follicles	Number of primordial follicles	Number of primary follicles	Number of secondary follicles
0	197.56 ± 15.31	83.78 ± 8.03	50.78 ± 6.66	37.00 ± 5.50
50	197.22 ± 24.80	93.89 ± 8.98	49.89 ± 9.27	35.00 ± 6.30
100	206.33 ± 22.64	92.89 ± 14.09	53.78 ± 6.34	41.44 ± 7.17
200	189.67 ± 21.04	84.33 ± 10.48	47.67 ± 5.84	34.89 ± 5.52

Data were expressed as mean ± SE

Fig. 3 Effect of in vivo treatment of metformin on ovulation pattern. Swiss albino mice were treated with 50, 100, and 200 mg/kg body weight of metformin daily for 4 weeks. After the completion of the treatment, mice were superovulated with PMSG (5 IU) and hCG (10 IU). The OCCs were collected from the oviduct in KSOM media and denuded by incubating OCCs in KSOM media supplemented with hyaluronidase (0.1 mg/mL). MII oocytes were collected and the data was expressed as mean ± SE of MII oocytes retrieved per animal ($n = 8$)

Fig. 5 The effect of in vitro treatment of metformin on the ROS level in MII oocytes. MII oocytes obtained from post-IVM of GV oocytes exposed to different concentrations of metformin (0, 10, 25, 50, 100, 250, and 500 μg/mL) were incubated with 10 μM DCFDA in DMEM for 30 min at 37 °C and 5% CO_2. The oocytes were washed and visualized under a fluorescent microscope. The images were captured and ROS intensity was measured using Q-capture software. The intensity corresponds to the ROS level in the oocytes and expressed as lum units. The data was represented as mean ± SE ($n = 150, 54, 58, 151, 148, 135,$ and 98 MII oocytes for 0, 10, 25, 50, 100, 250, and 500 μg/mL group respectively). [a]$P < 0.01$, [b]$P < 0.001$ compared with the control group

body weight at 4 weeks compared with other groups. However, when they reached 8 weeks, the weight was similar to that of control (Table 5).
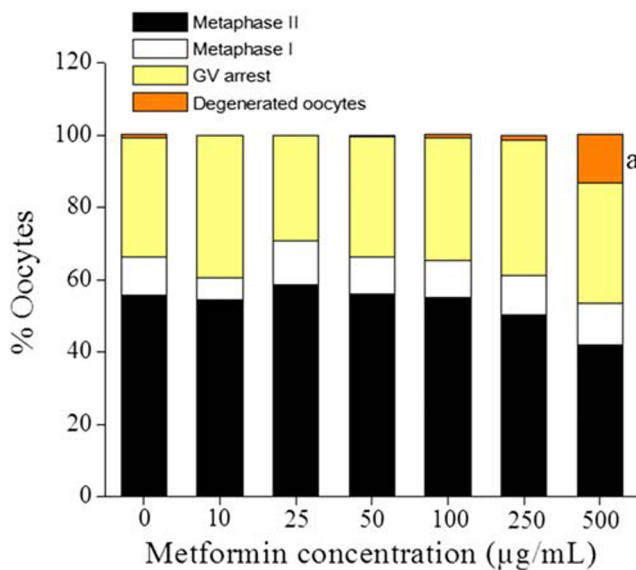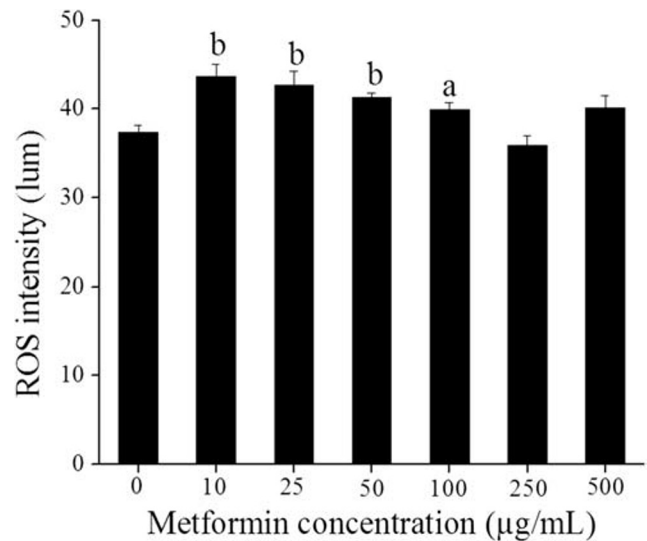

Fig. 4 Effect of in vitro treatment of metformin on oocyte maturation. GV oocytes were retrieved from the ovary by gently teasing the cortical region of the ovary and cultured in DMEM media supplemented with 1% non-essential α-amino acids, 1% ITS, 0.05% pyruvate, 0.3% BSA, and different concentrations of metformin (0, 10, 25, 50, 100, 250, and 500 μg/mL). Oocytes were incubated at 37 °C and 5% CO_2 for 24 h and the number of MII, MI, GV, and degenerated oocytes was recorded. The data was expressed as percentage ($n = 269, 99, 99, 269, 269, 269,$ and 234 GV oocytes for 0, 10, 25, 50, 100, 250, and 500 μg/mL group respectively). [a]$P < 0.01$ compared with the control group

Discussion

In the present study, we observed that the daily administration of metformin at 50, 100, and 200 mg/kg for 4 weeks did not alter the body weight and the blood glucose level. Our finding agrees with a previous report [24] which also demonstrated that metformin administration in healthy subjects did not alter glucose but decreased the post-prandial insulin secretion. Earlier studies have reported that the plasma level of metformin is highly variable in humans ranging from 54 to 4133 ng/mL which varies with the individual and dose of metformin administered [25]. Since the half-life of the compound is approximately 5 h [26], frequent administration of metformin is essential for maintaining the threshold level in blood to achieve reduction in blood glucose. In our study, daily administration of metformin did not cause any change in the liver weight but spleen weight was found to be decreased. Metformin administration to normal mice at high dose can induce metabolic stress which could be the reason for decreased spleen weight. Liver/spleen ratio is known to change in several pathological conditions such as liver cirrhosis [27] and biliary cirrhosis [28]. We observed a dose-dependent increase in liver/spleen (L/S) ratio post-metformin treatment. Similar finding was observed in an earlier study in which metformin increased liver to spleen (L/S) ratio in patients with non-alcoholic fatty liver disease [29]. This may be due to the anti-inflammatory function of metformin which is

Table 3 The effect of various doses of metformin on developmental potential of 2-cell-stage embryos cultured in KSOM

Metformin dose (mg/kg body weight)		0	50	100	200
No. of females		07	05	08	08
No. of 2-cell-stage embryos retrieved		25.29 ± 4.05	24.00 ± 3.21	22.13 ± 3.96	30.63 ± 5.34
No. of unfertilized oocytes		1.14 ± 0.86	1.00 ± 0.63	0.38 ± 0.26	0.63 ± 0.38
No. of fragmented oocytes		1.43 ± 0.81	1.00 ± 1.00	1.63 ± 0.60	3.00 ± 1.20
No. of 2-cell-stage embryos cultured		177	120	177	247
Morula rate (%)		99.44 (176)	100 (120)	98.87 (175)	93.12 (230)
Blastocyst rate (%)	3.5 dpc	66.67 (118)	71.67 (86)	59.32 (105)[e]	50.61 (125)[b,g]
	4.5 dpc	98.31 (174)	100 (120)	97.18 (172)	85.43 (211)[c,h,k]
Hatching rate (%)	3.5 dpc	5.08 (9)	4.17 (5)	0.57 (1)[a]	1.21 (3)[a]
	4.5 dpc	48.59 (86)	55.83 (67)	44.07 (78)	33.20 (82)[b,h,i]
Fragmented embryos at 4.5 dpc (%)		1.69 (3)	0	2.26 (4)	12.55 (31)[c,h,k]
Total cell number		108.12 ± 4.58	95.92 ± 2.42	95.28 ± 3.62	89.53 ± 2.45^{b}
Apoptotic index (%)		2.51 ± 0.45	1.98 ± 0.37	3.18 ± 0.44	1.87 ± 0.27

Data were expressed as mean $\pm$ SE and percentage. Data in parentheses represents the actual number of embryos at respective stages of development

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.0001$ vs control

[e] $P < 0.05$, [f] $P < 0.01$, [g] $P < 0.001$, [h] $P < 0.0001$ vs the 50 mg/kg group

[i] $P < 0.05$, [j] $P < 0.01$, [k] $P < 0.001$ vs the 100 mg/kg group

already reported. Even though there are several mechanisms by which metformin modulates immune response in auto-immune disorder [30, 31] and cancer condition [32, 33], altering the gut microbiome is considered to be more predominant [34].

The administration of metformin did not affect ovulation, follicle reserve, and endocrine profile. Metformin has shown to decrease the androgen production by thecal [6] and granulosa cells [35] due to decreased ATP and activation of AMPK which in turn inhibit anabolic process like steroidogenesis. Furthermore, a systemic review and meta-analysis revealed that metformin increased SHBG level leading to the further reduction in free testosterone [36]. Earlier studies have shown that metformin did not alter ovulation rate in women with PCOS [37] whereas other studies have reported adverse effects on

nuclear maturation in porcine and bovine oocytes [14, 15, 38]. In our study, in vivo exposure of metformin did not alter the number of ovulated oocytes following priming with PMSG and hCG. It has been shown that metformin causes arrest of bovine oocytes in germinal vesicle stage during in vitro maturation through activation of AMPK [14]. AMPK is a master regulatory protein which is generally activated when there is any stress in the form of elevated oxidative stress [39, 40] and increased AMP/ATP ratio [41]. However, other study has demonstrated that the inhibitory effect of metformin on nuclear maturation during IVM of porcine oocytes is not mediated through activation of AMPK [15]. In this study, Bilodeau-Goeseels et al. observed that compound C was not able to reverse the negative effect of metformin on oocyte maturation.

Table 4 The effect of in vitro exposure of various concentrations of metformin on developmental competence of 2-cell-stage embryos cultured in KSOM medium (24 embryos per group)

Metformin concentration (μg/mL)	2.5 dpc	3.5 dpc		4.5 dpc			
	6–8 cell rate (%)	Morula rate (%)	Blastocyst rate (%)	Blastocyst rate (%)	Hatching rate (%)	Total cell number	TUNEL index
0	95.83 (23)	95.83 (23)	70.83 (17)	91.67 (22)	29.17 (7)	83.04 ± 3.78	5.72 ± 0.77
10	100 (24)	100 (24)	79.17 (19)	95.83 (23)	33.33 (8)	90.09 ± 3.65	4.38 ± 0.76
25	95.83 (23)	95.83 (23)	50.00 (12)	83.33 (20)	12.50 (3)	88.30 ± 4.18	7.37 ± 1.27
50	100 (24)	100 (24)	54.17 (13)	95.83 (23)	25.00 (6)	71.82 ± 4.33	5.36 ± 1.63
100	100 (24)	100 (24)	41.67 (10)	95.83 (23)	8.33 (2)[a]	53.61 ± 2.90^{a}	2.05 ± 0.89
250	100 (24)	100 (24)	7.69 (2)[c]	7.69 (2)[c]	0[b]	-	-
500	37.50 (9)[a]	25 (6)[c]	0[c]	0[c]	0[b]	-	-

The developmental potential data were expressed as percentage (%). The total cell number and TUNEL index were expressed as mean $\pm$ SE

[a] $P < 0.01$, [b] $P < 0.001$, [c] $P < 0.0001$ compared with the control group

Table 5 Effect of various doses of metformin on the reproductive potential of female mice and the post-natal development in progenies

Metformin (mg/kg)	Mating efficiency (%)	Total number of litters	Average litter size	Body weight of progenies (g)			
				Parturition	2 weeks	4 weeks	8 weeks
0	86	93	8.50 ± 0.40	1.49 ± 0.01	5.92 ± 0.11	13.21 ± 0.30	23.60 ± 0.40
50	71	85	8.50 ± 0.45	1.43 ± 0.02	6.24 ± 0.09	14.67 ± 0.31[a]	24.15 ± 0.43
100	86	99	9.00 ± 0.41	1.43 ± 0.01[a]	6.23 ± 0.11	13.63 ± 0.31	24.81 ± 0.40
200	100	93	8.38 ± 0.43	1.46 ± 0.02	6.38 ± 0.11[b]	11.75 ± 0.41[a,c,d]	25.08 ± 0.47

The data were expressed as mean ± SE [$n = 12$ females and 12 males (1:1 ratio)] for all parameter except for mating efficiency which is expressed in percentage

[a] $P < 0.05$, [b] $P < 0.01$ compared with control; [c] $P < 0.001$ compared with 50 mg/kg; [d] $P < 0.001$ compared with 100 mg/kg

In vitro exposure data on oocyte maturation in our study indicates that at higher doses of metformin, the nuclear maturation is inhibited with higher incidence of oocyte degeneration and higher ROS level which might have resulted in activation of AMPK as reported in literature [40, 42].

Effect of metformin on oocyte and embryo development is contradictory. The major effect of in vivo administration of metformin was observed on the early embryo development where metformin significantly hindered blastocyst formation and decreased the hatching potential. An earlier study has demonstrated the presence of metformin transporters such as organic cation transporters (OCT) and multidrug and toxin extrusion proteins (MATE) in the endometrial epithelium of humans and rats suggesting the exposure of metformin to the embryos [43]. Our in vitro metformin exposure data agrees with an earlier study, where blastocyst rate was decreased drastically when frozen thawed 2-cell-stage embryos were exposed to metformin in vitro at a concentration of 100 μg/mL [23]. On the contrary, an in vitro study has used low concentrations of metformin ranging from 10^{-6} to 10^{-12} M [44] which are lower than what we used in the study, and these concentrations of metformin can inhibit the secretion of estradiol, progesterone, and androgen by human granulosa and thecal cells, whereas another study has claimed that metformin did not affect the development of embryos due to the lack of metformin transporter and unaltered AMPK level [45]. In vitro exposure to metformin revealed that 2-cell embryos were highly susceptible to metformin which is in agreement with a previous study where they compared 2-cell versus blastocyst exposure [46]. However, it was not clear whether similar degree of sensitivity is expressed at all stages of pre-compaction development. To understand this aspect, we assessed the effect of metformin on different stages of development (supplementary Table 1). The blastocyst forming potential was lowest in embryos exposed to metformin at 2-cell stage, and the sensitivity gradually decreased with their progression to 4, 6–8-cell, and morula stages. Even at the lowest concentration, metformin significantly decreased the blastocyst rate in embryos exposed at 2-cell stage suggesting its extreme sensitivity to metformin.

These observations provide clues on possible shift in their sensitivity to metformin post-embryonic genome activation. The adverse effect of metformin on embryo development could also be due to the direct inhibitory action of metformin on ETC which in turn affects the production of ATP [2, 3]. In addition, activation of AMPK is known to alter the expression of gap junction proteins which regulate the key events during compaction [16] and subsequent formation of blastocyst. This could be the reason for the arresting embryos at compaction stage post-exposure to metformin which is an AMPK activator. In addition, an earlier study has reported the reduction in stemness, proliferation, and metabolic pattern in mouse embryos exposed to metformin [17] which could be another cause for the developmental arrest of embryo.

The total cell number in blastocyst determines the quality of the blastocyst and is decreased at high-dose metformin indicating that high concentration of metformin affects the embryonic cell division. Further, delay in development of embryos exposed to metformin indicates its possible effect on cell cycle progression. Several studies done on tumor cells [47–49] and on keratinocytes [50] have shown that metformin induces G0/G1 cell cycle arrest and apoptosis and affects the proliferation. Since there are no reports regarding the effect of metformin on cell cycle during embryo development, the further studies need to be carried out in this direction.

Metformin freely crosses the placental barrier exposing a developing fetus to therapeutic concentration of the drug [51]. An earlier study revealed that metformin decreases the circulating insulin in women with PCOS [52] and insulin is known to inhibit hepatic SHBG synthesis [53]. Therefore, metformin-mediated increase in SHBG can cause reduction in testosterone and AMH affecting sexual differentiation and early gonadal development. In our study, we did not observe any change in pregnancy rate, litter size, and developmental anomalies in the progenies born. Earlier follow-up studies have shown that the children born to mothers with PCOS treated with metformin exhibited increased BMI, obesity, and altered metabolism at 4 and 9 years of age [54, 55]. However, body
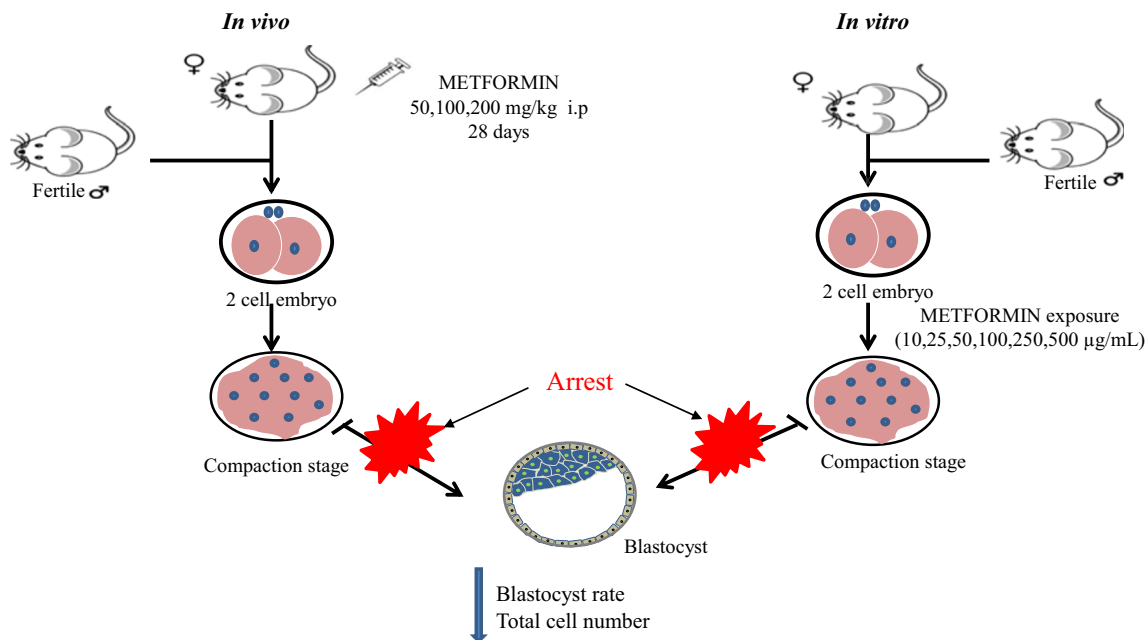
Fig. 6 Schematic representation indicating conclusion of the study. The metformin exposure at higher concentration both in vivo and in vitro in mice revealed significant adverse effects on the early embryo development by decreasing blastocyst rate and also arresting embryo at compaction stage

weight was decreased in litters at 4 weeks of post-natal life but it was comparable at 8 weeks when compared with control.

In conclusion, from the present study, it appears that metformin is a safe drug as far as ovarian function is concerned. However, the early embryo development seems to be severely affected especially at higher dose/concentration of metformin used in the present study (Fig. 6). The major limitation of the study is that we have not assessed the level of metformin in the blood or in the oviduct of metformin-injected mice which could have given us a hint about the appropriate concentration of metformin to be used for embryo toxicity assessment. The doses used for this study was based on an earlier report where the human embryos were exposed to metformin in the range of 10 to 100 μg/mL [23]. A systematic review has shown that the therapeutic plasma concentration can be as high as 90 μg/mL [56]. On the contrary, majority of the studies report much lower concentration of metformin in serum [25, 57, 58]. Even though our study demonstrates embryotoxic effect of metformin at higher doses, one may argue that the lowest dose used in our in vitro study (10 μg/mL) is much higher than the highest serum concentration reported in the literature. Therefore, further studies are essential to draw conclusive evidence to confirm that metformin at the therapeutic dose has embryo toxic effects. Future studies on assessing the possible effects of metformin on the function of key metabolic enzymes involved in early embryo metabolism, expression of gap junction proteins during early embryo development, and whether disruption of AMPK-mediated signaling cascades caused by metformin can be reversed by using AMPK inhibitor such as compound C can be important research avenues to explore. Therefore, even though metformin is considered to be safe and used

extensively in treating various human disorders, the results obtained from the present study draw attention about using it in fertility treatments routinely. This warrants further extensive research to establish the safety of the drug, especially during the early embryo development and fetal gonadogenesis.

Compliance with ethical standards

Animal handling and experimental procedures were done according to the institutional guidelines, and the study protocols were approved by the Institutional Animal Ethics Committee of Kasturba Medical College (IAEC/KMC/25/2014).

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