Electronic Journal of General Medicine
2022, 19(4), em381
e-ISSN: 2516-3507
https://www.ejgm.co.uk/

MODESTUM

Original Article

OPEN ACCESS

Oxidative stress and metformin: An in-vitro study on serum and primary human granulosa cell cultures

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Citation: Alam F, Abidi SH, Farooqi N, Jehan F, Rehman R. Oxidative stress and metformin: An in-vitro study on serum and primary human granulosa cell cultures. Electron J Gen Med. 2022;19(4):em381. https://doi.org/10.29333/ejgm/12037

ARTICLE INFO

Received: 20 Dec. 2021

Accepted: 11 Apr. 2022

ABSTRACT

Objective: To observe the effect of metformin on the oxidative stress (OS) levels and SIRT1 expression in primary human granulosa cell cultures from infertile females.

Methods: This cross-sectional study was conducted from August 2017-July 2019 on ten infertile patients. After written informed consent, the follicular fluid of these subjects was collected for establishing primary human granulosa cell cultures (HGCs), to test the capacity of metformin to reduce OS. The cultured HGCs were divided into (i) control: HGCs without any treatment, (ii) test1: HGCs induced with H_2O_2 (metformin absent), and (iii) test2: HGCs treated with metformin after H_2O_2 induction. Baseline OS (control group) was estimated, it was induced and measured by an increase in optical density (OD) (test1 & test 2). Finally, test2 was incubated with metformin (1 ml of 100 mmol/l concentration) and it effect on the levels of OS were determined by Mishra OS assay. Effect of metformin modulating SIRT1 expression in OS-induced cells was analyzed using quantitative-PCR.

Results: Age of female subjects was 32.04±2.29years and BMI was 27.61±2.15 kg/m^2. Test (OS induced) samples gave an OD of 0.28 (0.16-0.40) while control HGC samples gave an OD; 0.153 (0.09-0.23). Test cells showed significant reduction in ODs after metformin treatment. The relative expression of SIRT1 in metformin untreated and treated cells was found to be 61.5% and 80%, respectively.

Conclusion: Metformin was found to suppress OS in HGCs and increases the expression of SIRT1 in OS induced environment of primary cell cultures, suggesting a relationship between metformin, SIRT1 expression, and reduction of OS.

Keywords: antioxidants infertility, female, oxidative stress, primary human granulosa cell cultures, metformin, SIRT1

INTRODUCTION

Compromised ability to conceive or infertility has emerged as a serious health concern, affecting approximately 15% of couples worldwide and 23% of couples in Pakistan [1]. The possibility of conception is primarily dependent on the up keeping of not just the quantity but also the quality of the ovarian reserve [2]. The maintenance of the ovarian reserve is affected by multiple factors including physiological and genetic factors. Increased age of the female, contributes to ovarian aging owing to the increased level of redox activity [3].

Oxidative stress (OS) is a resultant of mitochondrial dysfunction, where it either fails to fight against the oxidants or the expression of the antioxidants is not sufficient [4]. Superoxide, hydrogen peroxides, hydroxyl ions, and NADPH oxidases are major forms of reactive oxygen species, and they are formed as by-products of cellular respiration during the electron transport chain reactions in the mitochondria. The same mitochondria possess antioxidants to counteract these harmful elements. When similar activity happens within the ovarian cells, there is a decrease in the fertility potential of oocytes [5]. Cellular redox activity is warranted to carry out the normal physiological functions of reproduction in males and females but the in-equilibrium disturbs the hormonal support as well as the fertility at the organ level by blocking the accomplishment and preservation of oocyte maturity [6]. Cellular damage, including DNA damage, is a common result of OS, which affects the oocyte maturation and the cleavage phase in the early embryonic stage [1]. The raised levels of OS markers are hypothesized to compromise the maturation of the nucleus and mitotic spindles of the maturation oocytes [7].

Metformin (generic name; dimethyl biguanide), extensively prescribed as a hypoglycemic drug reduces the process of hepatic gluconeogenesis [8]. Metformin also inhibits gluconeogenesis by obstructing mitochondrial redox shuttles and fructose-1,6-bisphosphatase-1 (FBP1) in the liver [9,10]. It is a drug of choice for reducing glucose levels and has also been

Abstract of this research paper is presented in the 37th ESHRE Conference, 26 June-1 July, 2021; and published in *Human Reproduction, 36*(Supplement_1). https://doi.org/10.1093/humrep/deab130.228

recognized as counteracting OS by various pathways [11]. Metformin is also proficient in reducing ROS by affecting its production, either by reducing the respiratory chain reactions within the mitochondria or by reducing NAD(P)H [12]. It also increases the glutathione content, in an attempt to reduce ROS [13] and also increases the expression of antioxidant genes, i.e., Nrf2 [14]. It also enhances mitochondrial biogenesis and deactivates apoptosis via the capases3 pathway [15]. It reduces the inflammation by activating adenosine-mono-phosphate (AMK), upregulating phosphatase and TENsin (PTEN) expression, reducing phosphatidylinositol 3-Kinase (PI3K)-protein kinase (Akt) and activating nuclear factor kappa B (NF-kB) and up-regulating the expression of PGC-1a, thus reducing the proliferation within the vessels [16]. Metformin induces insulin sensitization [10,17] thus is a suitable candidate to reduce metabolic symptoms in PCOS patients. Furthermore, it decreases OS and improves insulin resistance, dyslipidemia, and endothelial dysfunction in PCOS patients [18].

There have been certain studies done on PCOs patients which validate the potential benefit of metformin treatment in improvement of fertility after assisted reproductive treatment and further prevention of miscarriage and implantation failure in IVF cycles [19]. However, no study could establish the mechanistic role of metformin in improved gonadotropin ovulation induction outcomes [20]. This advocates metformin to offer much more than what is known. In our previous study it was proposed that further detailed genetic studies involving cell lines are demanded to validate the role of metformin in controlling adverse effects of OS on the gynecologic milleu and improvement in fertility potentials. This might surface a new venue for therapeutic increase in fertility and would possible be more cost-effective.

To the best of our information, no study has yet been conducted which focuses on the effect of metformin on oxidative stress in primary human granulosa cells of infertile females. Therefore, the rationale for this study is to identify the improvement of OS status with the administration of metformin in the oocyte microenvironment of females with various causes of infertility.

MATERIAL & METHODS

Recruitment of Subjects

It is a prospective cohort study using convenient sampling techniques, follicular fluid was collected from 10 infertile female patients undergoing egg retrievals for assisted reproductive technique (ART) at the Australian concept infertility medical centre (ACMIC). The fluid samples were collected after obtaining written informed consent from the study participants. The study was conducted after obtaining approval from the Ethical Review Committee of Aga Khan Hospital (AKU-ERC-2018-0557-601), and all relevant ethical guidelines were followed.

All patient fulfilling the criteria of primary infertility in the age group of 18 to 40 years were included in the study; while women with age more than 40, having BMI more than 30 kg/m^2, thyroid problems, gynecological tumors, hypertension, diabetes, congenital adrenal hyperplasia, androgen-secreting tumors, cushing syndrome, 21-hydroxylase-deficient non-classic adrenal hyperplasia, androgenic/anabolic drug use or abuse and women with serious general health status were excluded. Females on oral contraceptive pills and hormonal treatments or practicing any contraceptive measures were also excluded.

Collection of Follicular Fluid Samples

The follicular fluid was conducted with all aseptic measures and samples were transported on ice. Oocytes were retrieved 36±1 hours after ovulation induction on the 14th, 15th, or 16th day of controlled ovarian stimulation by vaginal ultrasound probe with 16G adapter and double-lumen oocyte aspiration needle (Cook Australia, Queensland, Australia) under short general anesthesia. During oocyte retrieval, follicular fluid was obtained from a dominant follicle (≥18 mm) from each ovary and aspirated into 10 mL tubes. The needle was retracted, and the content was emptied without the addition of a culture medium in the collection tubes. Efforts were made to minimize culture contamination. Maturity of oocytes was assessed by the presence of oocyte zona pellucida, nuclear maturity, presence, or the absence of the germinal vesicle or the first polar body. The cytoplasm was further examined for vacuoles or any other abnormalities in the texture of the ooplasm. Only normally fertilized (with two pronuclei and two polar bodies) were considered for eventual embryo transfer. Cleavage of embryos was confirmed after another 24-hour. Cleavage rate was assessed by counting the number of cells in the embryo on day three. Normal cleavage was considered by the presence of six to eight cells, slow cleavage by five cells or fewer, and accelerated cleavage with nine or more cells [21].

Isolation and Culturing of Human Granulosa Cells

To isolate human granulosa cells, follicular fluid was centrifuged at 3,500 rpm for 10 minutes. The supernatant was discarded and 2ml of 0.1% w/v of hyaluronidases enzyme (sigma H3506) was added to the pellet. After incubation for 15 minutes at 37 °C, the pellet was dislodged by pipetting and transferred to Corning® cell strainer, size 70µm filter, and centrifuged for 30 minutes at 450 ×g. After the centrifugation, the interface of the density gradient was collected and washed with 1× phosphate buffer saline pH 7.4. After washing, the pellet was re-suspended in 5ml of growth medium (Dulbecco's modified eagle's medium with 20% fetal bovine serum and 5% streptomycin) and transferred in a T-25 tissue culture grade corning flask, to allow the growth of human granulosa cells.

Induction and Estimation of Oxidative Stress in Human Granulosa Cells

The cultured human granulosa cell lines (HGCs; n=10) were grown for 24-48 hours up to 70-80% confluency. For our experimental assay, the cultured confluent cells were divided into three group in a 6-well plate: (i) control: HGCs without any treatment, (ii) test 1: HGCs induced with H_2O_2 (metformin absent), and (iii) test 2: HGCs treated with metformin after H_2O_2 induction.

For OS induction, HGCs were washed with FBS-free Dulbecco's modified eagle medium (DMEM). Dilution of H_2O_2 was made from a 30% stock solution into DMEM just before each experiment and 100 µM H_2O_2 solution was added to the cells [22]. The culture plates were incubated for 24 hours in a CO_2 incubator at 37°C. After 24 hours of incubation with H_2O_2, the levels of oxidation in the samples were analyzed via superoxide dismutase activity using the Mishra H.P method [23]. Briefly, 0.1 ml of cell line and media, 0.15 ml of ice-chilled

chloroform, and 0.75 ml of ethanol were added and mixed. At 3,000 rpm for 15 minutes the tubes were centrifuged, and the supernatant was collected. 1.0 ml of 0.1 M of carbonate-bicarbonate buffer (pH 10.2), 0.5ml of EDTA (0.6mM), and 0.5 ml epinephrine (1.8mM) was added per sample. Changes in absorbance were recorded at 480 nm. Baseline oxidative stress (in control group) was estimated using the same procedure except for H_2O_2 induction.

Treatment and Estimation of the Effect of Metformin on Reduction of Oxidative Stress

The H_2O_2 induced HGCs cells (n=10) were then incubated with 1 ml of 100 mmol/l concentration of metformin (Sigma, St Louis, MO, USA for additional 24 hours [24]. The effect of metformin on the levels of oxidative stress were also determined by Mishra oxidative stress assay as described above. The following formula was applied to assess the percentage difference of oxidative stress activity and the effect of metformin: OD test 2-OD blank)/ (OD test 1-OD blank) × 100 % (here, the test 2 group was the metformin treated cells and the test 1 were the H_2O_2 induced cells (metformin untreated) whereas, carbonate-bicarbonate buffer was taken as blank).

Measurement of Levels of SIRT1 in Metformin-Treated and Untreated Cells

We determined whether metformin was affecting SIRT1 levels/expression which can also be a factor in reducing OS. For this experiment, levels of SIRT1 were measured in test 1 and test 2 (untreated/treated with metformin) as mentioned above, using quantitative PCR employing SIRT-1 specific primers [25]. For the protocol, the reaction was prepared by mixing SYBR™ Green master mix with SIRT1 forward primer 5'-TGCTGGCCTAATAGAGTGGCA-'3 and SIRT1 reverse primer 5'-CTCAGCGCCATGGAAAATGT-'3 and template cDNA (extracted from cultured cells) and nuclease-free water making the volume up to 10 ul. This reaction was performed on bio-rad CFX96 touch real-time PCR detection system. The real-time PCR condition was programmed as follows: 95°C for 10min, then 40 cycles of 95°C for the 30s, 61°C for 60s, followed by a ramp from 50 to 95°C for the melting curve stage. Ct values obtained from the experiment were used to calculate the percent relative SIRT1 expression (relative % Cq) using the formula B/A*100, where B is the gene in question and A is the housekeeping gene.

Table 1. Demographic profile & fertility parameters of infertile female

Factors	Study (n=10)
Demographic	
Age (years)	32.02±2.29
BMI (kg/m^2)	27.61±2.15
Fertility profile	
Infertility duration (years)	3.67±0.2
Progesterone levels (nmol/L)	54.23±32.13
Follicle-stimulating hormone (IU/L)	5.59±2.19
Luteinizing hormone (IU/L)	6.62±2.51
Estradiol (pg/ml)	230±4.30

Note. Results are represented as mean±SE

Statistical Interpretation

The comparison between paired samples (metformin untreated cells with metformin treated cells) was conducted with a Wilcoxon signed ranks test to measure any significant difference between both groups. All statistical analyses were conducted using the IBM statistical package for the social sciences (IBM SPSS version 21; IBM Corp Inc, Armonk, NY).

Ethics Statement

The samples were collected after obtaining written informed consent from the study participants. The study was conducted after obtaining approval from the Ethical Review Committee of Aga Khan Hospital (AKU-ERC-2018-0557-601). All relevant ethical guidelines laid out by the Ethical Review Committee of Aga Khan Hospital were followed.

RESULTS

A total of 10 infertile females were recruited in this study according to the inclusion and exclusion criteria. Their demographic and fertility profiles are mentioned in **Table 1**.

Estimation of Oxidative Stress in Human Granulosa Cells

Human granulosa cells isolated from the patient's follicular fluid were grown for 48 hours up to 80% confluency. The results of the assay revealed that the average OD in control HGCs at baseline (no induction and no metformin treatment) was 0.1534 (0.09-0.23), while the average OD after oxidative stress induction in HGCs was 0.28 (0.16-0.40) (**Figure 1**). The

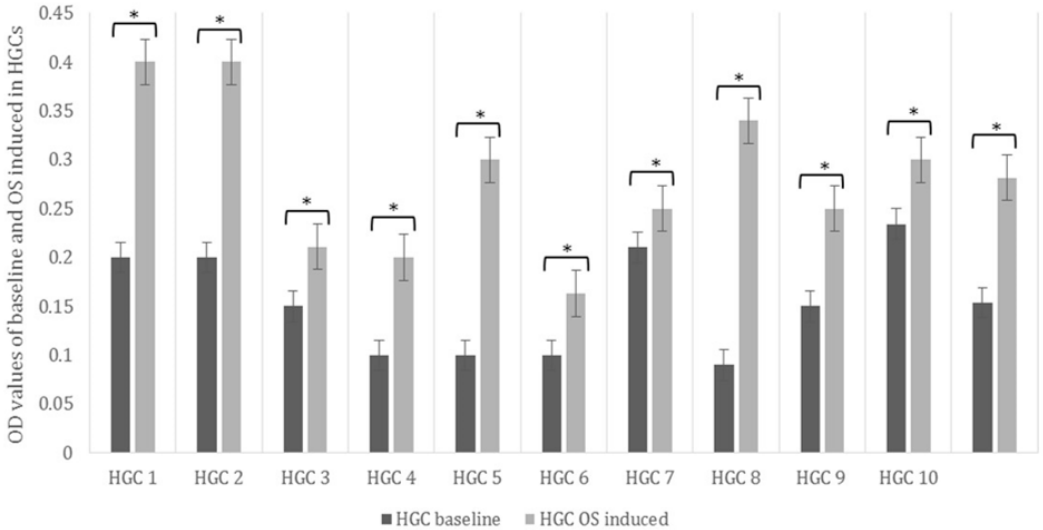

Figure 1. Optical densities at baseline and after OS induction in HGC samples

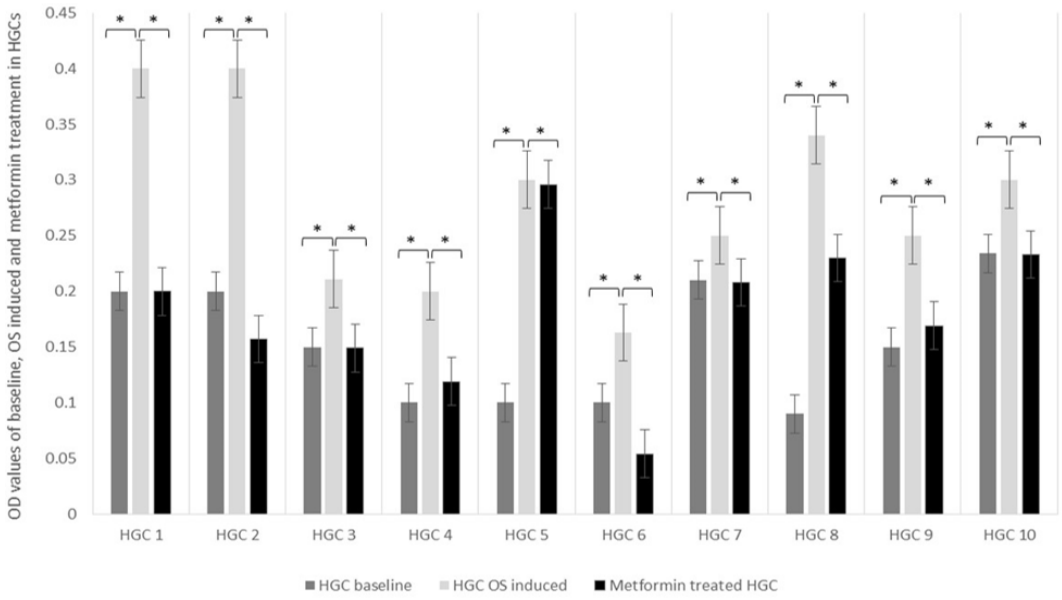

Figure 2. Optical densities at baseline, and for OS induced cells samples either untreated or metformin treated

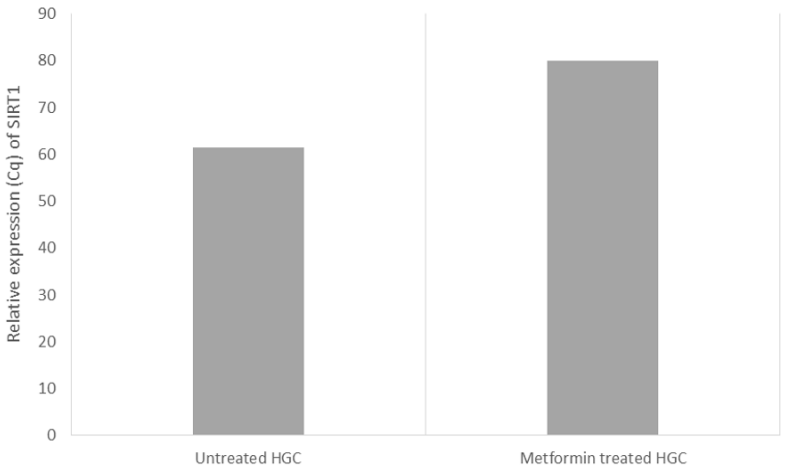

Figure 3. Percent relative expression of SIRT1 in metformin untreated and treated HGCs)

difference in OD before and after induction was statically significant (p=.005). The optical densities of the HGCs observed at baseline and after OS induction. HGC1-10 indicates the number of samples of human granulosa cells. The asterisk above the bar indicates significance between mean (p<0.05).

Effect of Metformin on Oxidative Stress in Human Granulosa Cells

The results of the assay after metformin treatment revealed a significant reduction in ODs, where the average OD in oxidative stress induced HGC dropped from 0.28 to 0.182 (0.05-0.30; p=.005) (**Figure 2**).

Measurement of Levels of SIRT1 in Metformin-Treated and Untreated Cells

In the next step, using qPCR, we determined whether metformin affected SIRT1 levels/expression which may also be a factor in reducing OS. The percent relative expression of SIRT1 was found to be 61.5% in untreated cells, while 80% in treated cells (**Figure 3**), suggesting that metformin induces SIRT1 expression in oxidative stress-induced cells and might lower the cellular OS via the SIRT1 pathway.

DISCUSSION

OS is a common biological event in polycystic ovarian syndrome (PCOS) and other causes leading to infertility. Our research group has established that OS constantly threatens the achievement and conservation of oocyte quality and quantity thus surfacing as a potential risk in the course treatment of infertility [26]. It has been observed that low levels of SIRT1 are also associated with increased OS leading to infertility [1]. In this study, we have explored reversal of OS by the introduction of metformin as has been proposed previously by our research group [13].

The role of metformin in female reproduction has been documented on account of its role in the restoration of regular menstrual cycles, initiation of ovulation, and hence increasing chances of fertility [27]. In an animal study where hyperandrogenism was taken into account for a direct cause of poor-quality oocytes, metformin treatment has proved to partially improve the impairment by increasing the fertilization rate, rate of blastocyst formation along with ATP production [28]. Our results on the role of metformin in improving OS are strengthened by literature which suggests the benefit of metformin in reducing the intrafollicular androgen level,

improvement of ovarian IR, and ovarian morphology in PCOS patient [29]. In addition to that, treatment with metformin improved the total number of oocytes and mature oocytes in the murine model [30]. We have observed that metformin reduced OS in a dose-dependent manner. These results comply with the recognized role of metformin in a dose-dependent fashion to upsurge NAD+/NADH ratio and SIRT1 sufficiency and actions [31-34].

We have observed that metformin decreased the oxidation activity in the stress-induced cells up to 85% and increased SIRT1 expression in OS-induced cells. A possible explanation of the role of metformin is by regulation of the NAD/NADPH ratio which increases the catalytic efficacy of SIRT1 [32]. Furthermore, the use of metformin with clomiphene emphasized its role in the down regulation of the SIRT3 gene in oocytes obtained from PCOS mice [35].

Metformin has shown to exert protective effects in many conditions. For example, cyclophosphamide is one of the chemotherapeutic agents prescribed in numerous childhood cancers such as leukemia, lymphoma, breast cancer, etc. [36]. The reduction in gonad toxicity with concurrent metformin treatment during cyclophosphamide therapy has highlighted its role in the preservation of ovarian function and fertility [36].

Assessment of OS levels can predict the utility of metformin before initiation of assisted reproductive treatment. A randomized control trial to see the efficacy of appropriate dose of metformin treatment in reduction of OS in both infertile male and females, can be a way forward. This will be a new discovery, first of its kind for human beings and will add to its established role in the treatment of diabetes, metabolic syndrome obesity, and PCOS. We expect that translation of this research will enable us to prescribe a cost-effective and easily available drug from 'bench-to-bedside' that will benefit patients as well as the community. The sequential work done by the group is thus expected to pave a pathway and develop plans for precision medicine leading to improvement in reproductive health, fetal and maternal well-being in a resource poor environment.

Limitation

The small sample size is the limitation of this study. The impact of metformin on HGCs cultures due to different causes of infertility could not be ascertained. A randomized control trial with a large sample size is recommended to confirm the cause-and-effect relationship.

CONCLUSION

Metformin suppresses oxidative stress in primary human granulosa cells and increases the expression of SIRT1 in OS induced environment in the primary cell cultures of human granulosa cells, therefore, metformin may be considered as a supplemental treatment option in infertile patients with infertility related to OS. This would be an inexpensive and easy approach to treat infertile couples before proceeding to ART. Proteomics analysis of follicular fluid can further reveal the effect of Metformin on the protein-protein interaction for improving OS leading to better fertility outcomes.

Author contributions: All authors have sufficiently contributed to the study, and agreed with the results and conclusions.

Funding: No funding source is reported for this study.

Declaration of interest: No conflict of interest is declared by authors.

REFERENCES

1. Alam F, Khan T A, Ali R, Tariq F, Rehman R. SIRTI and cortisol in unexplained infertile females; a cross sectional study, in Karachi Pakistan. Taiwan J Obstet Gynecol. 2020;59(2):189-94. https://doi.org/10.1016/j.tjog.2020.01.004 PMid: 32127136
2. Geraghty P. Each woman's menopause: An evidence based resource. Springer; 2022. https://doi.org/10.1007/978-3-030-85484-3
3. Holmes DT, Kline G. The endocrinology of aging. In: Winter WE, Holmquist B, Sokoll LJ, Bertholf RL, editors. Handbook of diagnostic endocrinology. Elsevier; 2021. p. 663-85. https://doi.org/10.1016/B978-0-12-818277-2.00018-2
4. Gualtieri R, Kalthur G, Barbato V, Di Nardo M, Adiga SK, Talevi R. Mitochondrial dysfunction and oxidative stress caused by cryopreservation in reproductive cells. Antioxidants (Basel). 2021;10(3):337. https://doi.org/10.3390/antiox10030337 PMid:33668300 PMCid:PMC7996228
5. Hardy MLM, Day ML, Morris MB. Redox regulation and oxidative stress in mammalian oocytes and embryos developed in vivo and in vitro. Int J Environ Res Public Health. 2021;18(21):11374. https://doi.org/10.3390/ijerph182111374 PMid:34769890 PMCid:PMC8583213
6. Nath B, Roy H. Antioxidants in female reproductive biology. In: Waisundara VY, editor. Antioxidants-Benefits, sources, mechanisms of action. 2021. https://doi.org/10.5772/intechopen.95937
7. Tatone C, Di Emidio G, Barbonetti A, et al. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. Hum Reprod Update. 2018;24(3):267-89. https://doi.org/10.1093/humupd/dmy003 PMid:29447380
8. LaMoia TE, Shulman GI. Cellular and molecular mechanisms of metformin action. Endocr Rev. 2021;42(1):77-96. https://doi.org/10.1210/endrev/bnaa023 PMid:32897388 PMCid:PMC7846086
9. Hunter RW, Hughey CC, Lantier L, et al. Metformin reduces liver glucose production by inhibition of fructose-1-6-bisphosphatase. Nat Med. 2018;24(9):1395-406. https://doi.org/10.1038/s41591-018-0159-7 PMid:30150719 PMCid:PMC6207338
10. Flory J, Lipska K. Metformin in 2019. JAMA. 2019;321(19):1926-7. https://doi.org/10.1001/jama.2019.3805 PMid:31009043 PMCid:PMC7552083
11. Podhorecka M. Metformin-its anti-cancer effects in hematologic malignancies. Oncol Rev. 2021;15(1):514. https://doi.org/10.4081/oncol.2021.514 PMid:33747367 PMCid:PMC7967492
12. Bhatti GK, Gupta A, Reddy PH, Bhatti JS. Translation of bioenergetics therapies: Challenges from bench to bedside. Clin Bioenerg. 2021;489-507. https://doi.org/10.1016/b978-0-12-819621-2.00022-x
13. Rehman R, Abidi SH, Alam F. Metformin, oxidative stress, and infertility: A way forward. Front Physiol. 2018;9:1722. https://doi.org/10.3389/fphys.2018.01722 PMid:30555352 PMCid:PMC6281994
14. Calabrese EJ, Kozumbo WJ. The hormetic dose-response mechanism: Nrf2 activation. Pharmacol Res. 2021;167:105526. https://doi.org/10.1016/j.phrs.2021.105526 PMid:33667690

15. Xie Y, Wang L, Khan M A, et al. Metformin and androgen receptor-axis-targeted (ARAT) agents induce two PARP-1-dependent cell death pathways in androgen-sensitive human prostate cancer cells. Cancers (Basel). 2021;13(4):633. https://doi.org/10.3390/cancers13040633 PMid:33562646 PMCid:PMC7914929
16. Deng M, Su D, Xu S, et al. Metformin and vascular diseases: A focused review on smooth muscle cell function. Front Pharmacol. 2020;11:635. https://doi.org/10.3389/fphar.2020.00635 PMid:32457625 PMCid:PMC7227439
17. Horakova O, Kroupova P, Bardova K, et al. Metformin acutely lowers blood glucose levels by inhibition of intestinal glucose transport. Sci Rep. 2019;9(1):6156. https://doi.org/10.1038/s41598-019-42531-0 PMid: 30992489 PMCid:PMC6468119
18. Uckan K, Demir H, Turan K, Sarikaya E, Demir C. Role of oxidative stress in obese and nonobese PCOS patients. Int J Clin Pract. 2022;2022:4579831. https://doi.org/10.1155/2022/4579831
19. Palomba S, Falbo A, La Sala GB. Effects of metformin in women with polycystic ovary syndrome treated with gonadotrophins for in vitro fertilisation and intracytoplasmic sperm injection cycles: A systematic review and meta-analysis of randomised controlled trials. BJOG. 2013;120(3):267-76. https://doi.org/10.1111/1471-0528.12070 PMid:23194199
20. Costello MF, Chapman M, Conway U. A systematic review and meta-analysis of randomized controlled trials on metformin co-administration during gonadotrophin ovulation induction or IVF in women with polycystic ovary syndrome. Hum Reprod. 2006;21(6):1387-99. https://doi.org/doi:10.1093/humrep/dei501 PMid:16449310
21. Ashraf M, Mustansir F, Baqir SM, Alam F, Rehman R. Changes in vitamin E levels as a marker of female infertility. J Pak Med Assoc. 2020;70(10):1762-6. https://doi.org/10.5455/JPMA.40329 PMid: 3159749
22. Upadhyay S, Vaish S, Dhiman M. Hydrogen peroxide-induced oxidative stress and its impact on innate immune responses in lung carcinoma A549 cells. Mol Cell Biochem. 2019;450(1-2):135-47. https://doi.org/10.1007/s11010-018-3380-2 PMid:29938378
23. Campos-Shimada LB, Hideo Gilglioni E, Fernandes Garcia R, Rizato Martins-Maciel E, Luiza Ishii-Iwamoto E, Luzia Salgueiro-Pagadigorria C. Superoxide dismutase: A review and a modified protocol for activities measurements in rat livers. Arch Physiol Biochem. 2020;126(4):292-9. https://doi.org/10.1080/13813455.2018.1520891 PMid: 30372625
24. Shentu X-C, Ping X-Y, Cheng Y-L, Zhang X, Tang Y-L, Tang X-J. Hydrogen peroxide-induced apoptosis of human lens epithelial cells is inhibited by parthenolide. Int J Ophthalmol. 2018;11(1):12-7. https://doi.org/10.18240/ijo.2018.01.03 PMid:29375984 PMCid:PMC5767651
25. Li X-H, Li D, Liu C, Zhang M-M, Guan X-J, Fu Y-P. p33ING1b regulates acetylation of p53 in oral squamous cell carcinoma via SIR2. Cancer Cell Int. 2020;20:398. https://doi.org/10.1186/s12935-020-01489-0 PMid:32831651 PMCid:PMC7436958
26. Alam F, Khan TA, Amjad S, Rehman R. Association of oxidative stress with female infertility-A case control study. J Pak Med Assoc. 2019;69(5):627-31. PMid:31105280
27. Jamilian H, Jamilian M, Foroozanfard F, Afshar Ebrahimi F, Bahmani F, Asemi Z. Comparison of myo-inositol and metformin on mental health parameters and biomarkers of oxidative stress in women with polycystic ovary syndrome: A randomized, double-blind, placebo-controlled trial. J Psychosom Obstet Gynecol. 2018;39(4):307-14. https://doi.org/10.1080/0167482X.2017.1383381 PMid: 28980870
28. Walters KA, Bertoldo MJ, Handelsman DJ. Evidence from animal models on the pathogenesis of PCOS. Best Pract Res Clin Endocrinol Metab. 2018;32(3):271-81. https://doi.org/10.1016/j.beem.2018.03.008 PMid:29779581
29. Raperport C, Chronopoulou E, Homburg R. Effects of metformin treatment on pregnancy outcomes in patients with polycystic ovary syndrome. Expert Rev Endocrinol Metab. 2021;16(2):37-47. https://doi.org/10.1080/17446651.2021.1889366 PMid:33634727
30. Pericuesta E, Laguna-Barraza R, Ramos-Ibeas P, et al. D-chiro-inositol treatment affects oocyte and embryo quality and improves glucose intolerance in both aged mice and mouse models of polycystic ovarian syndrome. Int J Mol Sci. 2020;21(17):6049. https://doi.org/10.3390/ijms21176049 PMid:32842637 PMCid:PMC7504697
31. Bost F, Rena G, Viollet B. Metformin: Beyond diabetes. Front Endocrinol (Lausanne). 2019;10:851. https://doi.org/10.3389/fendo.2019.00851 PMid:31866949 PMCid:PMC6909380
32. Cuyàs E, Verdura S, Llorach-Parés L, et al. Metformin is a direct SIRT1-activating compound: Computational modeling and experimental validation. Front Endocrinol (Lausanne). 2018;9:657. https://doi.org/10.3389/fendo.2018.00657 PMid:30459716 PMCid:PMC6232372
33. Kosgei VJ, Coelho D, Guéant-Rodriguez R-M, Guéant J-L. Sirt1-PPARS cross-talk in complex metabolic diseases and inherited disorders of the one carbon metabolism. Cells. 2020;9(8):1882. https://doi.org/10.3390/cells9081882 PMid: 32796716 PMCid:PMC7465293
34. Torres W, Nava M, Galbán N, et al. Anti-aging effect of metformin: A molecular and Therapeutical perspective. Curr Pharm Des. 2020;26(35):4496-508. https://doi.org/10.2174/1381612826666200716161610 PMid:32674728
35. Kamalipour F, Jalali H, Azarnia M. Comparison the effect of metformin and clomiphene citrate on sirtuin3 gene expression in the oocytes of mice with polycystic ovary syndrome. Iran J Pharm Res. 2020;19(4):160-8. https://doi.org/10.22037/ijpr.2020.113321.14236 PMid: 33841532 PMCid:PMC8019885
36. Huang C-C, Chou C-H, Yang Y-S, et al. Metformin: A novel promising option for fertility preservation during cyclophosphamide-based chemotherapy. Mol Hum Reprod. 2021;27(1):gaaa084. https://doi.org/10.1093/molehr/gaaa084 PMid:33543290 PMCid:PMC8494485