doi.org/10.36721/PJPS.2024.37.3.REG.663-667.1

Role of metformin in male oxidative stress infertility (MOSI): An *in vitro* experimental study

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Abstract: The study aimed to determine the *in-vitro* effect of metformin on total antioxidant capacity (TAC) of seminal samples of infertile male subjects. It was conducted from January to June 2022 on forty-four seminal plasma samples collected from male infertile patients, age ranging from 18 to 55 years. All 44 semen samples were treated as three distinct groups: (i) a control group (ii) a study group subjected to oxidative stress (OS) induction and (iii) a test group exposed to OS induction and subsequent treatment with metformin. OS was introduced by using commercially available 100µM hydrogen peroxide (H_2O_2) and incubated for twenty-four hours at 37ºC. After that 1 ml of 100 mmol/l concentration of commercially available Metformin (PHR 1331, CAS: 461-58-5) was administered to test group samples for additional 24h at 37ºC. Low levels of TAC were observed after OS induction in comparison to the control group (p=0.01). In test samples (after treatment with Metformin), a positive correlation of TAC with sperm count, normal sperm morphology and sperm motility were observed however, results were not significant. The antioxidant effect of Metformin was shown to improve the antioxidant capacity of OS induced samples and their sperm parameters in seminal plasma of infertile male subjects.

Keywords: Oxidative stress, metformin, infertility, sperm parameters.

INTRODUCTION

Infertility is the inability of a couple to perceive a viable pregnancy after one or more years of unprotected and frequent intercourse (Feferkorn and Tulandi, 2021). Male factor is usually the sole contributor in 20-70% of the infertility cases (de Vries *et al*., 2024). This may be due to impairment in semen concentration, normal morphology and/or motility (Harris *et al*., 2011). In addition to gonadal dysfunction, subjects with functional hypothalamus-pituitary-gonadal axis can also present with abnormal sperm parameters (idiopathic male infertility) (Agarwal *et al*., 2021, Esteves *et al*., 2024).

Different mechanisms are responsible for causing infertility in males. Oxidative stress (OS); an unevenness in reactive oxygen species (ROS) and antioxidants is the key causative factor affecting sperm quality and quantity (Hussain *et al*., 2023). Spermatozoa, ambassadors of male fertility are predisposed to the detrimental effects of OS and reactive oxygen species (ROS) due to greater proportion of poly unsaturated fatty acids (PUFA) in their cell membranes, limited antioxidant defense, redox imbalance, and impaired DNA repair system (Shahid *et al*., 2021). Free radicals break the DNA of the sperm and are responsible for lipid per oxidation (Khalid *et al*., 2023). Antioxidants play a vital role in neutralizing and eliminating free radicals, contributing to the positive impact of antioxidants on male infertility. Nevertheless, an excess of antioxidants could potentially lead to detrimental effects on the male reproductive system, thereby inducing male infertility (Ghosh *et al*., 2024). Total antioxidant capacity (TAC) assays have been widely employed in the analysis of biological samples to determine the levels of extra cellular non-enzymatic antioxidants (Silvestrini *et al*., 2023).

Metformin is an antioxidant, oral insulin-sensitizer, approved drug by Food and Drug Administration, which has been used for the last 50 years (Campbell, 2023). The role of metformin was studied on the testes of streptozotocin-induced diabetic rats with increase spermatozoa concentration, motility, and testosterone levels in comparison to non-treated rats (Zheng *et al*., 2023). Metformin is anticipated to enhance spermatogenesis through its influence on insulin resistance (IR), weight reduction and metabolic regulation (Tseng, 2022). The antioxidant effects of Metformin occur by the fractional restoration of the antioxidant enzymes (super oxide dismutase, catalase, and glutathione peroxidase), reduction in the level of oxidants (malonic aldehyde) and stabilization of Nrf2 factor expression for protection against ROS (Shpakov, 2021).

Metformin suppressed the basal and insulin-stimulated aromatase, which is required for transformation of

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androgens to estrogens, hence restored the ratio of testosterone to estradiol ratio and improved the spermatogenesis in cell culture experiments (Shpakov, 2021).

Rationale

The rationale for conducting this study on in 'vitro testing of metformin on OS' in semen samples emphasizes on the need to address the impact of OS on male infertility. The study will help to discover the potential benefits of metformin as a therapeutic option followed by controlled experimentation and *in vitro* testing. The results will have broader implications for reproductive health and gather new potential treatment options for controlled experimentation and precise measurement of oxidative stress markers Therefore, to improve fertility potential in male subjects by consideration of metformin as an adjuvant treatment therapy, we first aimed to determine the *in-vitro* effect of metformin on TAC of seminal samples of infertile male subjects.

MATERIALS AND METHODS

This study was conducted at the Department of Biological & Biomedical Sciences (DBBS), The Aga Khan University, Karachi (AKU) from January 2022 to June 2022. The ethical approval was acquired from the Ethics Review Committee (ERC No.2021-5405-19499) of AKU. This study was performed on 44 seminal plasma samples of male infertile subjects, aging from 18 to 55 years to assess TAC. The samples were collected in 15ml falcon tubes by masturbation in a closed environment at the Australian concept infertility Center (ACIMC) after taking consent from the subjects approaching the center. The semen analysis of the sample was assessed at ACIMC.

The detailed semen analysis report (SAR) and the samples were received at AKU in a temperature-controlled environment. The semen samples were centrifuged at 16,000 x g at 4ºC to separate and obtain seminal plasma which was aliquoted in 1.5ml micro tubes. Samples (44) were divided into three groups: baseline (control), study (OS induced without treatment) and test (OS induced and treatment with metformin).

Grouping

1. Control: For baseline results of TAC in semen samples
2. Study: Samples induced with OS without any treatment
3. Test: Samples induced with OS and treatment with metformin

Steps of procedure

Estimation of TAC

To detect the total non-enzymatic antioxidant capacity (TAC) in seminal plasma, the commercial kit (Cat. No E2199 Hu, standard curve range 0.3U/ml-90U/ml with a sensitivity of 0.14U/ml.) was used according to the manufacturer's manual. The sample and buffers were incubated together with the TAC-HRP conjugate in pre-coated plate for one hour. Following the incubation period, the wells were emptied, and a washing procedure was performed five times. Subsequently, the wells underwent incubation with an HRP enzyme substrate, resulting in the formation of a blue-colored complex through the enzyme-substrate reaction. Finally, with a stop solution to stop the reaction, the solution turned to yellow color. The optical densities (ODs) were measured spectrophotometric ally at 450nm in a micro plate reader within 10 minutes. TAC was determined in control group.

Induction of oxidative stress

Study and Test group samples were treated with 100µM hydrogen peroxide (H2O2). Incubated for twenty-four hours at 37ºC. TAC was estimated.

Metformin treatment

Test samples after induction of OS were incubated with 1ml of 100 mmol/l commercially available metformin kit (PHR 1331, CAS: 461-58-5) for additional 24 hours at 37ºC before estimation of TAC.

STATISTICAL ANALYSIS

The analysis of the concentration was performed by SPSS ver.23. The descriptive for quantitative variables were reported as Mean ± SD/ median (IQR) depending on the normality of the data. The normality of the data was assessed by Shapiro Wilk test of normality. The baseline, study and test groups were then compared for their total TAC. To determine the median TAC difference before and after treatment with metformin, a Wilcoxon sign rank test was used. Correlation analysis was performed by Spearmen's test to determine correlation between TAC and sperm parameters. A p value of less than 0.05 was considered significant throughout the study.

RESULTS

Table 1 shows the semen parameters (sperm count, sperm morphology and motility) of 44 infertile male subjects. The mean age of the participants was 35 ±5.5 years with a mean BMI of 26 ± 3.2 kg/m^2. We observed that the median sperm count was 42.5 (11) million sperm per ml, while median normal sperm morphology was 6% according to absolute Kruger criteria and median sperm motility was 24.5 (3.62) %. Comparison of TAC in samples at baseline (control), before and after administration of Metformin in OS induced samples study and test samples respectively shown in fig. 1.

Table 2 depicts the comparison of TAC in test samples after metformin treatment which gave a significant

Table 1: Descriptive statistics of male infertile subjects (n=44)

Variables	Values
Age (Years) mean ±SD	35.3 ± 5.5
BMI (Kg/m^2) mean ±SD	26.2 ± 3.2
Sperm count (mill/ml) median (IQR)	42.5 (11)
Normal sperm morphology (%) median (IQR)	6 (3.25)
Sperm motility (%) median (IQR)	24.5 (3.62)

Table 2: Comparison of total antioxidant capacity before and after administration of metformin in male infertile subjects (n=44)

	TAC (ng/ml) (Study Group)	TAC (ng/ml) Test Group (After Treatment	*P* value
Number of samples	44	44	
Median	1.07	1.82	
Percentiles (25)	0.43	0.44	0.001*
(50)	1.07	1.82	
(75)	1.77	2.93	

*Significant at p value < 0.05 by using Wilcoxon sign rank test

Table 3: Correlation of sperm parameters with total antioxidant capacity before and after administration of metformin in male infertile subjects

Variables	Sperm Parameters					
	Sperm Count		Normal Sperm Morphology		Sperm Motility	
	R	*P*	*R*	*P*	*R*	*P*
Before treatment with Metformin	-0.158	0.305	-0.187	0.225	-0.109	0.48
After treatment with Metformin	0.154	0.318	0.182	0.237	0.14	0.365

*Significant at p value < 0.05 and r value = 1 means a perfect positive correlation and r value = -1 means a perfect negative correlation by using Spearmen's test

improvement in tested samples. A negative correlation of sperm parameters with TAC was observed before metformin administration. However, after administration of Metformin (test samples) a weak positive correlation of sperm count, normal sperm morphology and sperm motility with TAC was observed results, however, were not significant as shown in table 3.

DISCUSSION

This study was aimed to evaluate changes in the TAC levels induced by ROS and metformin administered infertile male subjects. TAC levels are often higher infertile male subjects as compared to infertile male subjects (Silvestrini *et al*., 2023). In our experiment, TAC levels significantly decreased after induction of ROS in comparison to the baseline levels. This supports the fact that major cause of male infertility are sperm dysfunctions due to oxidative injuries caused by increase in ROS and decreased total antioxidant defenses in a seminal plasma (Vessey *et al*., 2021). The decrease in antioxidants further educes the fertilizing potential of male subjects which can cause infertility (Ghosh *et al*., 2024). Smoking and obesity are the modifiable risk factors in our population which significantly reduce sperm count and normal morphology by increase in OS (Abdelwahed *et al*., 2023).

Metformin, recognized for its cost-effectiveness and efficacy as an antioxidant, plays a significant role in activating Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) (Zhang *et al*., 2023). The antioxidant role of Metformin on microenvironment of human granulosa cells (HGCs) for fertility restoration in females was also studied by our research group (Rehman *et al*., 2020). In another experiment administration of metformin decreased the induced OS activity to 89% in Human Granulosa Cells (HGCs) and 76% in serum samples of infertile females. We have also hypothesized the probable effect of Metformin on OS and male fertility (Rehman *et al*., 2018).

In the present experiment, we observed that though not significant, yet metformin induced samples showed improvement in their TAC which is supported by literature (Feferkorn and Tulandi, 2021). In our results, we found that metformin significantly improved levels of TAC in ROS induced samples (p=0.0001). Moreover, a negative correlation observed in OS induced sperm parameters before metformin administration were improved to some extent after metformin administration. Our results are supported by previously conducted studies related to the effect of metformin on male infertility. Metformin inhibited the destructive effects of microwave-

radiation on sperm motility and sperm energy by preventing OS and apoptosis (Men *et al*., 2023). In another study the synergistic effect of metformin and Orlistat improved the testicular functions, hormonal levels and antioxidant enzyme levels of obese rates by reducing free radical formation and inflammation experimentally induced obesity (Hamza and Alsolami, 2024).

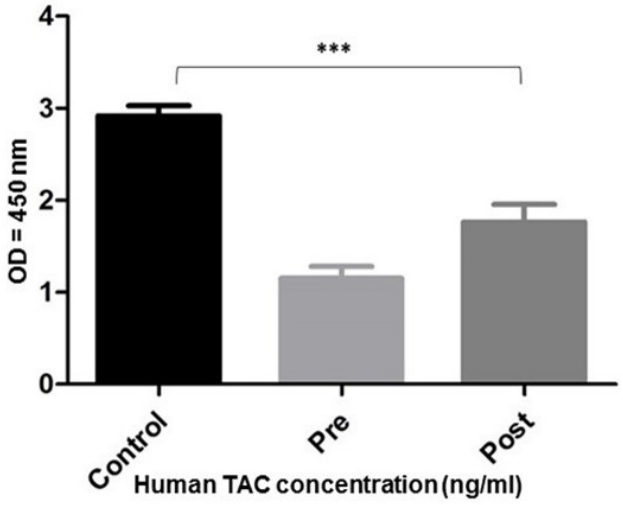

Fig. 1: Comparison of Total Antioxidant Capacity in control samples (baseline) and after induction of oxidative stress(OS). Pre and post represent (before and after) administration of Metformin in OS induced samples depict results of study and control samples ***: represents p value <0.001, OD is optical density

In our study, we used an external source to support or increase the scavenging activity of antioxidants by testing infertile seminal plasma with controlled amounts of metformin. With this we can propose that metformin alone or in combination can improve the sperm parameters. However, more randomized control trials needed to be done to validate these results.

It was a small sized study. It was not possible for us to estimate sperm parameters after administration of metformin due to logistic issues. However, to the best of our knowledge, the research on OS and male infertility in Pakistan was undertaken only by our research group.

CONCLUSION

Metformin had an antioxidant role and tended to improve semen parameters. The results give a valuable insight into the potential benefits of Metformin in addressing fertility issues, but further research is essential to solidify these findings and pave the way for potential clinical applications.

After further experimentation, Metformin can be used as a personalized medicine in the treatment profile of infertile male subjects especially with idiopathic infertility and for Assisted Reproductive Techniques (ART) based on its easy availability, affordability and effectiveness. To derive this aim, we will move forward, liaise with the relevant authorities and infertility centers to plan for human trials for introducing a cost-effective therapy with improvement in normal and assisted conceptions. This will be a new discovery, first of its kind for human beings and will add to established role of metformin in addition to its well-defined role in diabetes, obesity, and polycystic ovarian syndrome. It may lead to effective and safe infertility care within public health care structures in our country and other resource-poor countries.

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