Effects of Metformin on Experimental Varicocele in Rat

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Abstract

The aim of the current study was to determine effect of the Metformin (MET) on histopathologic evaluation and antioxidant enzyme activity in experimental varicocele-induced rat. Sixty rats were randomly divided into 6 experimental groups. The group 1 (control) had no received any medications and surgery. The group 2 (Sham) had no received any medications, abdominal cavity was opened but no varicocele-induced. The group 3 (Varicocele): abdominal cavity was opened, Varicocele-induced and no any medications applied. Group 4 abdominal cavity was opened, Varicocele-induced and animal received 25mg/kg of MET for 42 days. The groups 5 and 6 were similar to group 4, except animals received 50 and 100 mg/kg of MET, respectively. At the end of the days 21 and 42, rats were euthanized and left testis was removed for histological analysis and superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx) and total antioxidant status (TAS) levels. According to the results, dose dependent difference detected on testis damage grade in MET treated groups compared with varicocele group (P<0.05). No difference observed between 25 and 50 mg/kg of the MET (P>0.05). Tissue MDA levels significantly increased in varicocele rat (P<0.05) while MET (25, 50 and 100 mg/kg) in a dose dependent manner decreased varicocele-induced MDA (P<0.05). Experimental varicocele significantly decreased SOD activity compared to control group (P<0.05). Administration of the MET (25, 50 and 100 mg/kg) significantly increased tissue SOD activity in varicocele rat (P<0.05). MET (25, 50 and 100 mg/kg) in a dose dependent manner increased GPx activity in varicocele rat (P<0.05). No difference observed on MDA, SOD and GPx levels between 25 and 50 mg/kg groups (P>0.05). These findings suggested MET treatment had benefit effect against varicocele.

Keywords: Metformin, Histologic evaluation, Antioxidant, Varicocele, Rat
INTRODUCTION

Varicocele is an abnormal vascular dilatation of pampiniform plexus. Clinically, they are found more commonly on the left side, although there is wide variation among the reported prevalence of bilateral varicocele. Most anatomic research has been conducted on the internal spermatic vein and varicocele formation; however, there are some data to suggest that dilated external spermatic veins can also contribute to primary or recurrent varicocele (Masson and Brannigan, 2014). The pathophysiology of testicular damage in varicocele is not completely understood, however, gross testicular alterations associated with varicocele are well documented. Their findings suggest that varicocele cause a progressive decline in fertility and can continue to induce impairment of spermatogenesis, despite prior fertility (Celik-Ozenci et al., 2006; Masson and Brannigan, 2014).

Metformin (N, N-dimethylbiguanide, MET) is an orally administered biguanide that is commonly prescribed for the treatment of type 2 diabetes (Soraya et al., 2012). Besides its glucose-lowering effect, MET positively affects vascular endothelial function and atherosclerosis. MET inhibits pro-inflammatory response and apoptosis in human vascular wall cells (Schramm et al., 2011). The association of the varicocele with male infertility derives back to the first century AD when Celsius reported a link between dilated scrotal veins and testicular atrophy (Masson and Brannigan, 2014). Despite several investigations were done on role of MET against cell death (Oishi et al., 2014), scarce information exists on role of MET with male infertility. In a study it is reported as MET possesses a non-genomic action, it could be an interesting molecule to treat sperm which can improve fertility (Bertoldo et al., 2014). It is reported MET improves semen characteristics in men with metabolic syndrome (Morgan et al., 2011). Spermatozoa are particularly vulnerable to oxidation of their lipid plasma membranes due to the composition of fatty acids in the membrane and the relative inability to combat against oxidative stress. It is reported MET has the ability to decrease reactive oxygen species (ROS) (Bertoldo et al., 2014).

The mechanism of MET action has been studied primarily in the context of diabetes, and it is poorly understood. In the context of atherosclerosis, MET inhibits NF-κB activation and decreases C-reactive protein levels and it inhibits the inflammatory response (Hirsch et al., 2013). Although many infertile people have varicocele, its relationship with male infertility still remains unexplained (Zhang et al., 2006). Based on the literature review, no report exists on role of the MET on experimental unilateral varicocele-induced rat. So, the aim of the current study was to determine effect of the MET on histopathologic evaluation and antioxidant enzyme activity in experimental varicocele-induced rat.
MATERIAL AND METHODS

Study animals
To survey the protective role of MET on spermatozoa characteristics in experimental unilateral Varicocele-induced rat, sixty male Wistar rats (230-250 g) were allocated into 6 treatment groups. The rats were housed individually under standard laboratory conditions according to European community suggestions for laboratory animals at a temperature of 21±2°C, relative humidity of 55-60% and a 12 h light period. All animals had free access to chow pellets and fresh water. All experimental procedures were carried in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann, 1983). Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the current laws of the Iranian government.

Experimental creation of Varicocele
All surgical procedures were performed under anesthesia by intraperitoneal (i.p.) injection of 60 mg/kg ketamine hydrochloride 10% and 10 mg/kg xylazine hydrochloride 2%. The upper left abdominal quadrant was approached through a midline laparotomy incision. Herein, the renal and adrenal veins and the left spermatic vein inserts into the left renal vein. With a midline incision the left renal vein was exposed and after fine dissection of proximal left renal vein, left renal vein was tied using a silk suture (4-0) (Turner et al., 2001; Sahin et al., 2005). At the point of medial to insertion of the adrenal and spermatic vain into the renal, a metal probe (diameter ranging from 0.4-0.85 based on size of renal vein) was placed. The ligature was made around the probe, then probe removed and the vain allowed expanding within the boundary of ligature. This procedure leads to decrease renal vain diameter to one half. The midline incision of the abdominal wall and the anterior abdominal muscles were repaired, separately (Celik-Ozenci et al., 2006).

Study design
Sixty rats were randomly divided into 6 experimental groups (n=10). The group 1 (control) had no received any medications and surgery. The group 2 (Sham) had no received any medications, abdominal cavity was opened but no varicocele-induced. The group 3 (Varicocele): abdominal cavity was opened, Varicocele-induced and no any medications
applied. Group 4 abdominal cavity was opened; Varicocele-induced and animal received 25mg/kg of MET for 42 days. The groups 5 and 6 were similar to group 4, except animals received 50 and 100 mg/kg of MET, respectively. At the end of the days 21 and 42, rats were euthanized with an overdose injection of pentobarbital (300 mg/kg, i.p.), peritoneum opened and left testis was removed for further investigations.

**Histologic evaluation**

The tissue was fixed in Bouin’s solution (7.5 mL saturated picric acid, 2.65 mL glacial acetic acid, and 2.5 mL 7% formaldehyde), post-fixed in 70% alcohol, and embedded in paraffin blocks. A tissue section (5µm) were obtained, deparaffinized, and stained with hematoxylineeosin. The testicular tissue was evaluated in random order with standard light microscopy by an observer who was unaware as to which group the rat had belonged. Then, testis tissue samples from the experimental rats were fixed at Bouin’s solution for complete fixation and processed for paraffin sectioning. A tissue section about 5µm thickness were taken and stained with hematoxylin and eosin [H & E]. The testis sections were graded numerically to assess the degree of histological changes associated with seminiferous tubule injury as previously described by Johnsen as bellow (Johnsen 1971):

- 10: complete spermatogenesis and perfect tubules
- 9: many spermatozoa present but disorganized spermatogenesis
- 8: only a few spermatozoa present
- 7: no spermatozoa but many spermatids present;
- 6: only a few spermatids present
- 5: no spermatozoa or spermatids present but many spermatocytes present
- 4: only a few spermatocytes present
- 3: only spermatogonia present
- 2: no germ cells present
- 1: neither germ cells nor Sertoli cells present

**Antioxidant activity**

Malondialdehyde is a standard to determine free radical damage. The detecting kit was purchased from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). Malondialdehyde was formed as an end product of lipid peroxidation and treated with thiobarbituric acid (TBA) to produce a colored product that was measured at 532 nm (Placer et al. 1966). The commercial kit was obtained from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). According to this method, GPx catalyses the oxidation of glutathione and in presence of glutathione reductase and NADPH, oxide glutathione
converts to reduced form by changes in oxidation of NADPH to NADP⁺ in absorbance at 340 nm (Paglia and Valentine, 1967). SOD detecting kit was purchased from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). The role of SOD is to accelerate the dismutation of the toxic superoxide radical (O₂), produced during the oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye detectable at 505 nm (Paoletti and Mocali, 1990). Nicotinamide adenine dinucleotide oxidation was measured at 340nm and expressed as U/mg tissue. The total antioxidant status detecting kit was obtained on the basis of suppression in color production which was measured at 600nm and expressed as mmol/ml (Miller et al., 1993).

**Statistical analysis**

Shapiro-Wilk tests were used for normality of the obtained data. Then parametric data analyzed by one-way analysis of variance (ANOVA) using SPSS 24.0 and expressed as mean values ± standard error of mean (SEM). The differences between groups analysed using Duncan Multiple Range Test. The histopathological scores analyzed by KruskaleWallis test. P<0.05 was considered as significant differences between groups.

**RESULTS**

The effect of different levels MET on score for assessing in experimental testicular varicocele-induced rat 21 and 42 days post-surgery are presented in figures 1 and 2. As seen in figure 1, varicocele group had the lowest testis damage grade compared to the other groups (P<0.05). The testis damage grade was higher in varicocele group compared to the control group (P>0.05). Administration of the MET in a dose dependent manner improved testis damage compared with varicocele group (P<0.05). No difference observed between 25 and 50 mg/kg of the MET (P>0.05).

Effect of different levels MET (25, 50 and 100 mg/kg) on tissue values of MDA, SOD, GPx and TAS in experimental testicular varicocele-induced rat on day 42 is presented in table 1. According to the results tissue MDA levels significantly increased in varicocele rat (P<0.05) while MET (25, 50 and 100 mg/kg) in a dose dependent manner decreased varicocele-induced MDA (P<0.05). No difference observed on MDA levels between 25 and 50 mg/kg groups (P>0.05). Experimental varicocele significantly decreased SOD activity compared to control group (P<0.05). Administration of the MET (25, 50 and 100 mg/kg) significantly
increased tissue SOD in varicocele rat (\(P<0.05\)). No difference observed on SOD levels between 25 and 50 mg/kg groups (\(P>0.05\)). Different levels of the MET (25, 50 and 100 mg/kg) in a dose dependent manner increased GPx activity in varicocele rat (\(P<0.05\)). No difference observed on GPx levels between 25 and 50 mg/kg groups (\(P>0.05\)). MET (25, 50 and 100 mg/kg) had no significant effect on TAS levels on testicular varicocele-induced rat (\(P>0.05\)).

**DISCUSSION**

According to the results, dose dependent difference detected on testis damage grade in MET treated groups compared with varicocele group. No difference observed between 25 and 50 mg/kg of the MET. As seen in the current study, dose dependent difference detected on testis damage grade in MET treated groups compared with varicocele rats. As observed in the histological results, testis section of left testis in varicocele rats showing degenerated seminiferous tubules. Testis section of left testis in the metformin (25 mg/kg) followed by varicocele rats on day 21 showing seminiferous tubules with few spermatocyte and interstitial cells. Also, Metformin (50 mg/kg) lead to seminiferous tubules with few spermatocyte and interstitial cells between tubules. Metformin (100 mg/kg) improved varicocele injury which lead to many normal seminiferous tubules with few spermatocytes. In a recent report, Erdem et al., (2019) revealed oral gavages of the MET (300 mg/kg per day for 8 weeks) improved spermatogenesis, seminiferous tubule integrity and reduced apoptotic activity as manifested by the decreased expression of cleaved caspase 3 in rats with varicocele which our results were similar to this report. MET improved the semen parameters related to its effects on weight loss, increased testicular weight and reduced testicular cell apoptosis (Yan et al., 2015). On the other hand, Tartarin et al., (2012) reported MET at concentration 10 times higher than therapeutic levels decreased testosterone secretion and the number of Sertoli cells in rats when it was administered during pregnancy. Faure et al. (2016) reduction in testicular weight and testosterone level were observed in 6-week-old chickens treated with metformin for 3 weeks.

As observed in the current study, tissue MDA levels significantly increased in varicocele rat (\(P<0.05\)) while MET (25, 50 and 100 mg/kg) in a dose dependent manner decreased varicocele-induced MDA. Experimental varicocele significantly decreased SOD activity compared to control group. Administration of the MET (25, 50 and 100 mg/kg) significantly increased tissue SOD activity in varicocele rat. MET (25, 50 and 100 mg/kg) in a dose
dependent manner increased GPx activity in varicocele rat. Sperm membranes contain large amounts of unsaturated fatty acids (USFAs) which provide fluidity, a process that is necessary for membrane fusion (Hwang and Lamb, 2012). Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and the antioxidants that scavenge surplus free radicals (Hwang and Lamb, 2012). ROS are natural products of cellular metabolism which, in physiological amounts, are essential requirements of spermatozoa for sperm processes leading to successful fertilization, such as capacitation, hyperactivated motility and acrosomal reaction (Agarwal et al., 2014). A correlation detected among varicocele and semen oxidation where elevated ROS levels leads to diminished antioxidant capacity in the semen of varicocele-induced animal (Hsieh et al., 2006). These changes lead to abnormal sperm function and the infertility (Masson and Brannigan, 2014). A correlation between the increasing percentage of motile thawed spermatozoa stimulated by MET and the best success of the IVF in our conditions was observed (Bertoldo et al., 2014). Several papers have already described that during freezing and thawing, cell organelles, such as mitochondria can undergo injury via a considerable reduction in high membrane potential (Bertoldo et al., 2014).

MET therapy has been shown to normalize total and free testosterone and may therefore explain the beneficial effect of MET on semen parameters in oligo-terato-asthenozoospermic men (Morgante et al., 2011). It is reported MET has positive effect on proliferation and migration human umbilical vein endothelial cells. Recently, new mechanism(s) supported for the effect of MET but the accuracy of them still controversial (Esfahanian et al., 2012). For instance, Zhou et al., (2001) suggested that most of the beneficial effects of MET are mediated through its ability to activate the AMP-activated protein kinase (AMPK). Various biological effects have been attributed to the activation of AMPK by metformin. It interferes with the action of the mammalian target of rapamycin (mTOR) that functions as part of the cellular signaling processes regulating cell growth, cell proliferation, cell motility, transcription and protein synthesis. Perhaps, some effects of MET mediate via this pathway. However, identification of these effects needs further investigations. Vascular endothelial growth factor reduced apoptosis in varicocele-induced rats by decrease caspase-3 positive cells (Tek et al., 2009).

Sperm in the epididymis are vulnerable to oxidative damage during maturation and the storage stage. It has been proven that ROS have a key effect on sperm maturation and capacitation, and high ROS production leads to sperm dysfunction (Hsieh et al., 2006). Seminal plasma is endowed with frequent enzymatic antioxidants that include SOD, GPx,
MDA (Fingerova et al., 2007). So, the neutral levels of ROS are critical for normal fertilization, capacitation, hyperactivation and motility (Agarwal et al., 2009). Varicocelectomy reduce ROS levels increase in the antioxidant capacity of semen in infertile men (Masson and Brannigan, 2014). However, this also makes spermatozoa vulnerable to ROS attack. Seminal fluid is an important source of antioxidants in semen, as the lack of cytoplasm and DNA compaction in spermatozoa leaves very little room for translation or for antioxidant defenses. Lipid peroxidation has also been associated with a decrease in sperm motility (Agarwal et al., 2008).

MET stimulate lactate production which play key role in germ cells development and anti-apoptotic effect in Sertoli cells (Ghasemnejad-Berenji et al., 2018). It is reported, anti-apoptotic effect of the MET mediates via caspase-3 in rat testis (Erdem et al., 2019). The findings of the present study are consistent with previous studies that MET reduced apoptosis in testis with varicocele (Erdem et al., 2019). Cryopreservation in the presence of MET, increase of viability without effect of lipid peroxidation compared to control group in mouse spermatozoa (Bertoldo et al., 2014). The discrepancy between biochemical and protective actions of MET is not an isolated observation. Cisplatin-induced functional and histological nephropathy was not prevented by MET in a rat model in vivo, although MET significantly attenuated drug-induced lipid peroxidation and reactive oxidant species and preserved enzymatic and non-enzymatic antioxidants (Sahu et al., 2013). In contrast, MET prevented experimental gentamicin-induced nephropathy in the rat (Morales et al., 2010).

CONCLUSION

In conclusion these findings suggested MET treatment had benefit effect against varicocele. MET prevents the progression of varicocele-induced infertility by decrease elevated MDA and free radical scavenging activity through increase SOD and GPX levels in varicocele-induced rat.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

References


Mortality and cardiovascular risk associated with different insulin secretagogues compared with metformin in type 2 diabetes, with or without a previous myocardial infarction: A nationwide study. Eur Heart J 32,1900-1908.


Figure 1. histological score for assessing in testis associated with seminiferous tubules injury after 21 days experimental varicocel rat. The control group I had no received any medications and surgery. The Sham group had no received any medications; abdominal cavity was opened but no varicocele-induced. The Varicocele group: abdominal cavity was opened, Varicocele-induced and no any medications applied. Metformin (25 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 25mg/kg of Metformin for 42 days. Metformin (50 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 50 mg/kg of Metformin for 42 days. Metformin (100 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 100 mg/kg of Metformin for 42 days. Different letters (a-d) in each column indicate significant differences between treatments (P<0.05).

Figure 2. histological score for assessing in testis associated with seminiferous tubules injury after 42 days experimental varicocel rat. The control group I had no received any medications and surgery. The Sham group had no received any medications; abdominal cavity was opened but no varicocele-induced. The Varicocele group: abdominal cavity was opened, Varicocele-induced and no any medications applied. Metformin (25 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 25mg/kg of Metformin for 42 days. Metformin (50 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 50 mg/kg of Metformin for 42 days. Metformin (100 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 100 mg/kg of Metformin for 42 days. Different letters (a-d) in each column indicate significant differences between treatments (P<0.05).
Figure 3. Testis section of left testis in control and sham rats on day 21 showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules. H & E: hematoxylin and eosin.

Figure 4. Testis section of left testis in varicocel rats on day 21 showing degenerated seminiferous tubules (arrow) and loss of spermatogenesis (H&E). H & E: hematoxylin and eosin.
Figure 5. Testis section of left testis in the metformin (25 mg/kg) followed by varicocele rats on day 21 showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules. H & E: hematoxylin and eosin.

Figure 6. Testis section of left testis in the metformin (50 mg/kg) followed by varicocele rats on day 21 showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules. H & E: hematoxylin and eosin.
Figure 7. Testis section of left testis in the metformin (100 mg/kg) followed by varicocele rats on day 21 showing many normal seminiferous tubules (arrow) with few spermatocyte (Arrow head). H & E: Hematoxylin and eosin.

Figure 8. Testis section of left testis in control and sham rats on day 42 showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules. H & E: Hematoxylin and eosin.
Figure 9. Testis section of left testis in varicocel rats on day 42 showing degenerated seminiferous tubules (arrow) and loss of spermatogenesis (H&E). H & E: hematoxylin and eosin.

Figure 10. Testis section of left testis in the metformin (25 mg/kg) followed by varicocle rats on day 42 showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules. H & E: hematoxylin and eosin.
Figure 11. Testis section of left testis in the metformin (50 mg/kg) followed by varicocle rats on day 42 showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules. H & E: hematoxylin and eosin.

Figure 12. Testis section of left testis in the metformin (100 mg/kg) followed by varicocle rats on day 42 showing many normal seminiferous tubules (arrow) with few spermatocyte (Arrow head). H & E: hematoxylin and eosin.
Table 1. Effect of different levels metformin on tissue values of Malondialdehyde, Superoxide dismutase, Glutathione peroxidase and total antioxidant status on day 42 in experimental testicular varicocele-induced rat

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg tissue)</th>
<th>GPx (U/mg tissue)</th>
<th>TAS (mmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111.02 ± 1.24 a</td>
<td>5.32 ± 0.13 a</td>
<td>6.24 ± 0.26 a</td>
<td>16.15 ± 1.54</td>
</tr>
<tr>
<td>Sham</td>
<td>110.15 ± 1.35 a</td>
<td>5.18 ± 0.20 a</td>
<td>6.17 ± 0.33 a</td>
<td>16.11 ± 1.21</td>
</tr>
<tr>
<td>Varicocele</td>
<td>175.05 ± 2.32 a</td>
<td>1.25 ± 0.11 d</td>
<td>2.35 ± 0.16 d</td>
<td>13.21 ± 1.62</td>
</tr>
<tr>
<td>Metformin (25 mg/kg)</td>
<td>165.02 ± 2.25 b</td>
<td>1.26 ± 0.16 c</td>
<td>2.17 ± 0.23 c</td>
<td>13.22 ± 1.14</td>
</tr>
<tr>
<td>Metformin (50 mg/kg)</td>
<td>158.23 ± 2.13 b</td>
<td>3.34 ± 0.21 c</td>
<td>3.42 ± 0.16 c</td>
<td>14.26 ± 1.27</td>
</tr>
<tr>
<td>Metformin (100 mg/kg)</td>
<td>120.36 ± 1.24 c</td>
<td>4.27 ± 0.18 b</td>
<td>4.54 ± 0.11 b</td>
<td>13.11 ± 1.18</td>
</tr>
</tbody>
</table>

The control group had no received any medications and surgery. The Sham group had no received any medications; abdominal cavity was opened but no varicocele-induced. The Varicocele group: abdominal cavity was opened, Varicocele-induced and no any medications applied. Metformin (25 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 25 mg/kg of Metformin for 42 days. Metformin (50 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 50 mg/kg of Metformin for 42 days. Metformin (100 mg/kg) group: abdominal cavity was opened; Varicocele-induced and animal received 100 mg/kg of Metformin for 42 days. MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase, TAS: total antioxidant status. Different letters (a-d) in each column indicate significant differences between treatments (P<0.05).