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Protective effects of modafinil administration on testicular torsion/detorsion damage in rats

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ABSTRACT

Testicular torsion is a pathological condition which leads to sever scrotal pain and ischemia. After surgical reperfusion, oxidative stress factors cause to germ cell apoptosis. Thus, adjuvant therapy to surgery should be useful to decrease of ischemia/reperfusion (I/R) injury of testis. Modafinil, a drug to treat sleepiness, has been indicated to have anti-inflammatory effects. The aim was to evaluate the efficiency of modafinil administration after reperfusion surgery in a rat model of testicular torsion/detorsion (T/D). Male wistar rats were divided into three groups and each group contained 10 animals. To induce torsion right testis was rotated 720° clockwise and was left for 1 h. Modafinil group received modafinil (10 mg/kg) once daily intraperitoneally for 7 days after the surgery and the control group received physiologic saline once daily intraperitoneally for 7 days after the surgery. Thereafter, MDA, IL-1β and TNF-α levels and histopathological changes were investigated. MDA, IL-1β and TNF-α levels significantly increased in T/D group compared to the control group ($^{**}P < .01$ and $^{***}P < .001$, respectively). Moreover, modafinil administration significantly reduced these values compared to T/D group ($^{\#}P < .05$ and $^{\#\#}P < .01$, respectively). Histopathological changes such as degeneration in germinal cells were detected in testis T/D group of rats whereas modafinil administration prevented degeneration in germinal cells, edema and hemorrhage compared with T/D group. In conclusion, administration of modafinil after reperfusion surgery had protective role on testicular torsion in rat and reduced ischemia/reperfusion cellular injury via anti-inflammatory and decrease of oxidative stress.

1. Introduction

Testicular torsion is a surgical emergency that occurs when the spermatic cord twists. This pathological condition reduces blood supply and lead to sever scrotal pain and ischemia (Testicular torsion).

If surgery is performed within 6 h, it is possible to rescue the testes by 90% but after 24 h it reduced to 0% (Mellick et al., 2019). After manual reperfusion, some oxidative stress factors such as reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and reactive nitrogen species (RNS) including nitric oxide and release of pro-inflammatory cytokines will be increased, metabolic acidosis intra cellular calcium overload, dysfunction of mitochondria and lipid peroxidation occur that result germ cell apoptosis (Beheshtian et al., 2008; Shimizu et al., 2016). Indeed, following the testicular torsion, blood circulation is severed which damaged tissue and after detorsion, blood flow restored and cascade of events such as ROS and RNS production, lipid peroxidation and proinflammatory cytokines release such as Interleukin 6 (IL-6) and TNF-α occurred which resulting to extra tissue injury (Shimizu et al., 2016).

So, finding of agents as an adjuvant therapy to surgical procedures to decrease of ischemia/reperfusion (I/R) injury of testis is important.

Modafinil, Fig. 1, a nonamphetamine central nervous system (CNS) stimulant, is indicated for narcolepsy, shift work sleep disorder and obstructive sleep apnea (Lillicrap et al., 2016). It is also useful in

Abbreviations: CAT, catalase; iNOS, inducable nitric oxide synthase; IL, interleukin; I-R, ischemia–reperfusion; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor alpha; CNS, central nervous system.

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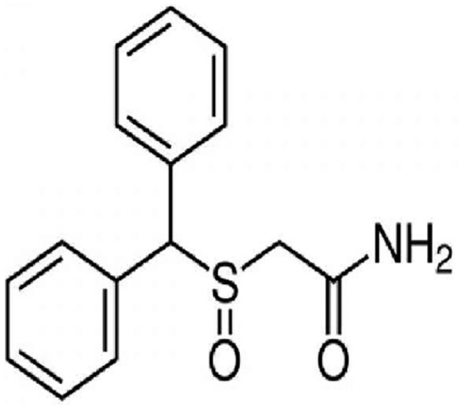

Fig. 1. Chemical structure of modafinil.

Parkinson's disease, multiple sclerosis to reduce fatigue (Sheng et al., 2013). Some studies indicated modafinil has anti-inflammatory effects (Brandt et al., 2014; Han et al., 2018; Raineri et al., 2012). Han et al. have demonstrated that modafinil not only decreased release of pro-inflammatory IL-6, TNF-α and IFN-γ, but also increased secretion of anti-inflammatory cytokines IL-4 and IL-10. They also have shown modafinil inhibited Akt/NF-κB pathway in apoE-deficient mouse and had a therapeutic effect for atherosclerosis (Han et al., 2018). In another study, researchers evaluated modafinil reduced expression of iNOS and COX-2 in LPS stimulated murine BV-2 microglia cell line exerting better anti-inflammatory effect than aspirin (Jung et al., 2012). Another investigation has shown that sleep disorder promoted pro-inflammatory and reduced anti-inflammatory cytokines and also promoted the number of activated microglial cells, but treatment with modafinil prevented all of these effects and has protective role in cognitive performance (Wadhwa et al., 2015). Researchers evaluated modafinil had a neuroprotective effect on cerebral ischemia and anti-apoptotic effect on cortical neurons via several mechanisms such as decreasing of LDH, Glut1, Glut3, PFK-1 levels and promoting PDH activity which prevents from lactic acidosis (Abbasi et al., 2019). Modafinil also, elevates the expression of BDNF and increases neuronal cell proliferation in rat dentate gyrus (Sahu et al., 2013). Modafinil pretreatment reduced IL-1β gene upregulation induced by LPS in mice brain and due it, modafinil inhibited depressive-like behavior induced by LPS in mice (Zager et al., 2018).

The purpose of this study was to evaluated the anti-ischemic features of modafinil administered before ischemia in a rat model of testicular torsion/detorsion (T/D). It is important to find an agent which can be useful as an adjuvant therapy before surgery to decrease testicular damage.

2. Material and methods

2.1. Animals

The present study was done on male Wistar rats weighing 220–250 g (8 weeks old) (Tehran university of medical sciences, Iran). Animals were kept under standard laboratory condition containing temperature: 24 ± 1 °C, humidity: 55 ± 10%, lighting: 12-h light/dark cycle with free access to standard animal chow and water. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985) (Code: 1538). This investigation has been supported by Tehran University of Medical Sciences and Health Services, Tehran, Iran.

2.2. Animal preparation and experimental testicular T/D procedure

The rats were divided into three groups and each group contained 10 animals. The rats were anesthetized with intraperitoneal injection of ketamine HCl (50 mg/kg) and chlorpromazine (25 mg/kg). To induce torsion right testis was rotated 720° clockwise and was left for 1 h and fixed by according to method described by Turner et al., 2004. After that, the testis was counter-rotated to the normal position (detorsion) and returned to the scrotum and connecting the gubernacular stumps with a 4–0 silk suture. Then, 7 days after detortion, animals were anesthetized by ketamine HCl (60 mg/kg i.p.) and xylazine (5 mg/kg i.p.), euthanized by decapitation and right testes of them were removed. Some of testes were kept in 10% formalin for histopathological analyses and the others were maintained in −80 °C for biochemical assays. To evaluate the effect of chronic administration of modafinil on torsion/detorsion procedure, a group of animals were received 10 mg/kg (Andersen et al., 2010) modafinil intraperitoneally (obtained from Sigma, St. Louis, MO, USA), dissolved in physiologic saline and also, once daily intraperitoneally for 7 days after the surgery. Control group as basal normal data.

2.3. Malondialdehyde assay

Malondialdehyde (MDA) is formed from lipid peroxidation of polyenic fatty acids. MDA is a marker of lipid peroxidation in tissues. MDA accumulation was measured by method as described by Ohkawa et al. briefly, animals were decapitated and testis tissues were removed and to make 10% (*w/v*) homogenate testes were homogenized in 1.15% KCl. 0.1 of homogenate was mixed with 0.9 ml of 1.8% sodium dodecyl sulfate (SDS), 1.5 ml of aqueous solution of TBA and 1.5 ml of 20% acetic acid solution (pH 3.5). The solution was heated (95 °C, 60 min), and cooled, then 5 ml *n*-butanol/pyridine (15:1, *v/v*) was added. After shaking, the mixture was centrifuged (4000 rpm, 10 min). The absorbance of the supernatant was measured at 532 nm. Data are expressed in μmol/g of total protein. 1,1,3,3-Tetramethoxypropane (TMP) was used as a standard.

2.4. Tumor necrosis factor alpha assay

TNF-α assay kit (Biosource, Camarillo, CA) was used to measure tissue TNF-α. The testes were homogenized in cold PBS and centrifuged (14,200 ×*g*, 30 min). Supernatants were used to TNF-α assay. Briefly, 50 μl of each sample or standard (bovine serum albumin) were added into a 96-well plate precoated with TNF-α antibody. 50 μl anti-TNF-α solution was added to each well and incubated for 90 min at room temperature. Afterwards, the wells were washed with wash buffer. Then, 100 μl of streptavidin peroxidase was loaded to each well (45 min incubation at room temperature), and washed with PBS. Followed by sequential addition of stabilized chromogen (100 μl, 20 min) and stop solution (100 μl). Thereafter, the optical density was analyzed at λ = 450 nm.

2.5. Interleukin 1 beta assay

Mouse IL-1 beta ELISA Kit (Abcam, Cambridge, UK) was used to measure tissue IL-1β. The testes were homogenized in cold PBS and centrifuged (14,200 ×*g*, 30 min). Supernatants were used to IL-1β assay. 100 μl of each sample or standard were added into wells and incubated for 2.5 h at room temperature. Afterwards, the solution was discarded and washed 4 times with 300 μl wash solution. 100 μl Biotinylated IL-1 beta Detection Antibody was loaded to each well and incubated for 1 h at room temperature with gentle shaking. Then, the solution was discarded and washed 4 times with 300 μl wash solution. 100 μl HRP-Streptavidin solution was added to each well and incubated for 45 min at room temperature with gentle shaking. After discarding of the solution and washing 4 times with wash buffer, 100 μl TMB

substrate reagent was loaded and incubated 30 min at room temperature in the dark with gentle shaking. Finally, 50 µl stop solution was added and immediately optical density was read at 450 nm in a spectrophotometer.

2.6. Histopathological study

For testicular morphological analysis, testicular tissues from each group were collected 7 days after reperfusion. The samples were fixed in 10% formalin for 24 h, dehydrated in paraffin wax and were cut in 5 µm sections by a microtome (RM2235 Rotary Microtome). Then were deparaffinized and stained with hematoxylin and eosin (H&E). The stained slides were observed for lesions under light microscope (original magnification, ×100). The sample was evaluated according to the 4 level grading scale of Cosentino's score. Grade 1: normal tissue and regularly organized germ cells; Grade 2: testicular damages with less organized; Grade 3: testicular damages with disordered, desquamation of germ cells and less distinction in seminiferous tubule borders; Grade 4: testicular damages with extremely germ cell necrosis; Two separate analyses were carried out.

2.7. Statistical analysis

All data were shown as mean ± SEM and analyzed using SPSS software (version 22) (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by post hoc Tukey's test was used to evaluate the differences between groups. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Biochemical assays

3.1.1. MDA, TNF-α and IL-1β levels

Fig. 2. illustrates the MDA levels in control, modafinil treated (Mod + T/D) and T/D groups. MDA value significantly elevated in T/D group compared to the control group ($^{**}P < .01$). Treatment with modafinil significantly reduced MDA level in Mod + T/D group compared to T/D group ($^{\#\#}P < .01$). There is no significant difference between the control group and Mod + T/D group. Fig. 3. shows the TNF-α levels in control, Mod +T/D and T/D groups. In T/D group TNF-α level significantly increased compared to control group ($^{***}P < .001$).

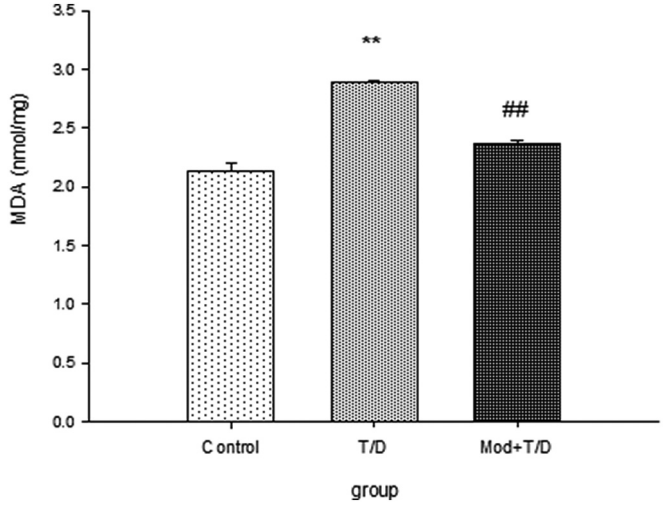

Fig. 2. Effects of Torsion/Detorsion and administration of modafinil on MDA levels of testis tissue.
$^{**}P < .01$ compared to the control group.
$^{\#\#}P < .01$ compared to T/D group.

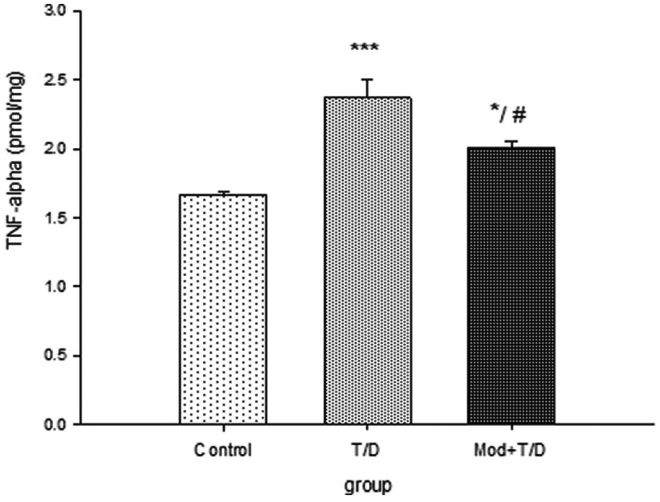

Fig. 3. Effects of Torsion/Detorsion and administration of modafinil on TNF-α levels of testis tissue.
$^{***}P < .001$ compared to the control group.
$^{*}P < .05$ compared to the control group.
$^{\#}P < .05$ compared to T/D group.

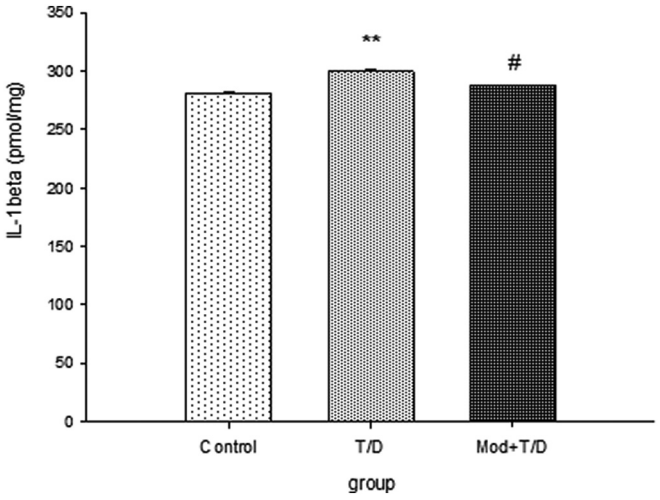

Fig. 4. Effects of Torsion/Detorsion and administration of modafinil on IL-1β levels of testis tissue.
$^{**}P < .01$ compared to the control group.
$^{\#}P < .05$ compared to T/D group.

Treatment with modafinil significantly decreased TNF-α level in Mod + T/D group compared to T/D group ($^{\#}P < .05$). there is also a significant difference between the control group and Mod + T/D group ($^{*}P < .05$). Fig. 4. shows the IL-1β levels in control, Mod +T/D and T/D groups. In T/D group IL-1β value significantly increased compared to control group ($^{**}P < .01$). Treatment with modafinil significantly decreased IL-1β level in Mod + T/D group compared to T/D group ($^{\#}P < .05$). There is no significant difference between the control group and Mod + T/D group.

3.2. Histopathologic assay

Fig. 5. indicates sections from testes of animals in three groups (control, Mod +T/D and T/D). Control group showed normal spermatogenesis and the histological structure of cell layers were reserved normal. However, several lesions including desquamation and degeneration in germinal cells were observed in testis T/D group of rats. Modafinil treated rats (Mod +T/D group) showed nearly normal

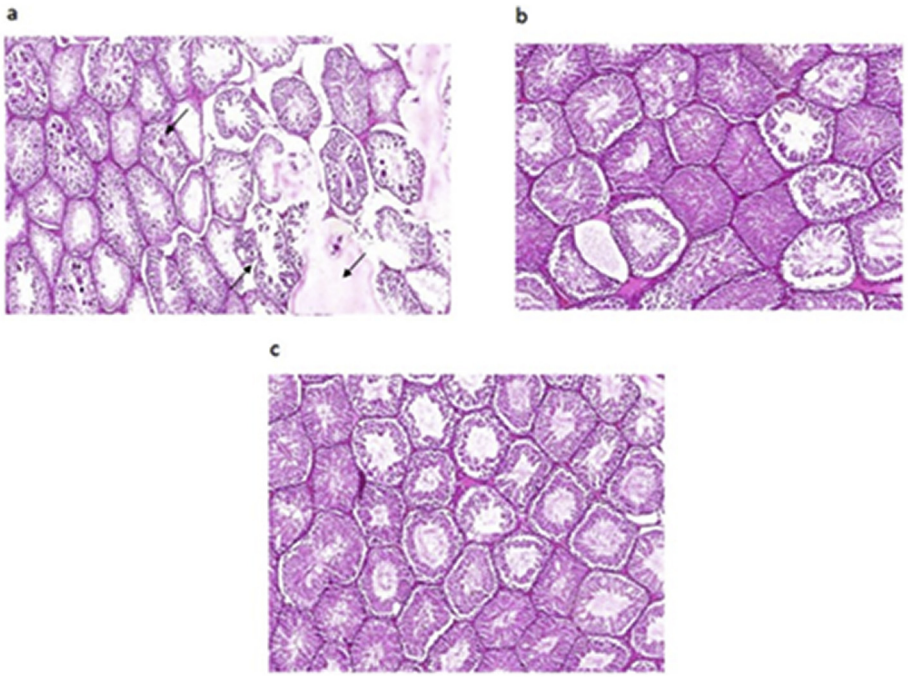

Fig. 5. Histopathological lesions were observed in testis sections of (a) torsion/detorsion operated group (T/D) which shows germinal cell degeneration, disorganization and interstitial edema (arrows). (b) Modafinil treated group (modafinil + T/D) and the control group (c) showed similar normal histopathological features (original magnification, ×100).

Table 1
Histopathological lesions in testis of experimental groups.

Histopathological lesions	Testis torsion/ detorsion (T/D group)	Modafinil treated (Mod +T/D group)
Germinal cell degeneration	+ +	–
Disorganization of germ cells	+	–
Desquamation in germ cells	+ +	+
Interstitial edema	+	–
Hemorrhage	+	–

structure. Some histopathological lesions in testis of groups explained in Table 1.

4. Discussion

Testicular torsion is an emergency and pathologic state requiring immediate surgery (Kostakis et al., 2017). If testicular torsion lasts for more than a few hours, it can hurt the testicle, and an impaired testicle must be removed (Ghasemnejad-berenji et al., 2018). After surgical procedure, it is not clear that the function of testes is completely preserved. Thus, it is essential to find out some strategies and therapeutic applications as adjuvant treatment in surgical detorsion (Kazaz et al., 2019). The aim of surgery is to restore blood flow and to correct testis perfusion. After surgical detorsion, overproduction of free radicals like ROS and over-expression of proinflammatory cytokines such as TNF-α and IL-1β occurs. Proinflammatory cytokines are responsible for neutrophil recruitment to the testicle tissue (Turner et al., 2004). Elevated level of MDA after testicular torsion/detorsion is a marker of high level of lipid peroxidation which causes damage to cell membrane and induces apoptosis in cells (Parlaktas et al., 2014). In present study, the effects of modafinil on T/D of testis of rats were evaluated. Now, for the first time, we report that modafinil had protective role against T/D damage in the rat testis via significant decrease of MDA when compared to T/D group. Modafinil reduced proinflammatory cytokines, TNF-α and IL-1β compare to T/D group which decreased testicular injury.

Modafinil, which is FDA approved for narcolepsy, has been illustrated to have anti-inflammatory effects and prevents neuroinflammation. An in vivo study on mouse model of atherosclerosis showed modafinil inhibits secretion of pro-inflammatory cytokines such as IL-6, TNF and IFN-γ and also, increases release of anti-inflammatory cytokines like IL-4 and IL-10. Modafinil suppressed proliferation of macrophages via inhibitory effect on Akt/IKK-alpha/NF-kappaB signaling pathway (Parlaktas et al., 2014). Another study indicated modafinil reduces morphological alterations in the astrocytes and microglia in the hippocampus of sleep-deprived rats and inhibited the activation of microglial cells and prevented neuroinflammation in rats (Wadhwa et al., 2015). A study on focal cerebral ischemia in rats illustrated modafinil administration decreased the edema, infarct volume, gliosis and apoptosis and had antioxidant properties which reduced the brain injury (Abbasi et al., 2019). Another observation showed modafinil and its analogues reduced free radical production and have positive effect on cognition (Fong, 2018).

Our results indicate, that 720° testicular torsion for one hour causes to elevate in MDA level as well as TNF-α and IL-1β levels versus control group. In addition, desquamation and degeneration in germinal cells were observed in testis T/D group of rats. Intraperitoneal administration of modafinil once daily intraperitoneally for 7 days after the surgery significantly reduced degeneration in germinal cells and histopathological changes compared to T/D group.

An in vitro study on BV2 cells showed modafinil and its derivatives have anti-inflammatory effects on LPS-induced cells via reducing expression of pro-inflammatory enzymes such as iNOS and COX2 (Jung et al., 2012). Another study on LPS-induced depressive-like behavior in mice, illustrated pretreatment with modafinil prevents inflammation-related depression and CNS-infiltrating macrophages and inhibits depression induced by LPS (Zager et al., 2015).

According to numerous studies, after ischemia-reperfusion of testis several mechanism, such as activation of inflammatory mediators like cytokines, neutrophil infiltration and the production of NO and ROS which lead to damage the cell membrane and DNA (Granger and Kvietys, 2015; Gürocak et al., 2011). Several observations showed modafinil has inhibitory effect on production of ROS which has protective role in neurodegenerative diseases and coronary artery ischemia and reperfusion injury (Farhoudi et al., 2013; Gerrard and Malcolm, 2007).

Based on our results, we have evaluated administration of modafinil after reperfusion surgery reduced proinflammatory cytokines, TNF-α and IL-1β when compared to T/D group which decreased testicular injury. Additional studies of positive effects of modafinil in other

experimental models could show it as an adjunct to surgical reperfusion on human testicular torsion.

Declaration of competing interests

None.

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