EFFECT OF CANNABINOIDS ON TESTICULAR ISCHEMIA-REPERFUSION INJURY IN RAT

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Abstract- Anandamide is an endogenous ligand for cannabinoid receptors and has endothelial protective effect against ischemic preconditioning. The purpose of this study was to investigate the effects of cannabinoids on reperfusion injury due to testicular torsion-detorsion (T/D). A total of 36 adult male Sprague-Dawley rats were divided into 6 groups. Testicular ischemia was achieved by twisting the right testes 720◦ counters clockwise for 1 hour and reperfusion was allowed for 4 hours after detorsion. In baseline (normal) group, bilateral orchiectomies performed after anesthesia. Sham operated group was served as a control group. Torsion/detorsion group underwent 1 hour testicular torsion and 4 hours of detorsion. Anandamide (cannabinoid agonist) group received pretreatment with intraperitoneally anandamide 30 min before torsion. AM251 (CB1 antagonist) group, received intraperitoneally injection of AM251 45 min before torsion. Anandamid/AM251 (An/AM) group received administrations of AM251 45 min before torsion and anandamide 30 min before torsion. The ipsilateral malondialdehyde (MDA) level in T/D group were significantly higher versus control and base line groups. Ipsilateral MDA values in anandamid group were significantly lower than T/D and An/AM groups. There were also significant decreases in catalase activity in T/D group compared with control and base line groups. These values were significantly higher in cannabinoid group versus T/D and An/AM groups. Anandamide increased ipsilateral intratesticular antioxidative markers and decreased free radicals formation during reperfusion phase after unilateral testicular torsion, which was reflected in lesser testicular MDA level. Furthermore, the effects of anandamide were mediated via cannabinoid receptors, since AM251 could abolish these effects.

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Key words: Ischemia-reperfusion, testicular torsion, cannabinoid, anandamide, rat

INTRODUCTION

Testicular Torsion (TT) is a urologic emergency, requiring early diagnosis and surgical intervention to prevent testicular damage (1). Treatment of testicular torsion may cause further damage the testes. Reperfusion of ischemic tissue leads to sequence of events that injure the tissue. The injuries produced by reperfusion can be more severe than the injuries induced by ischemia (2). Ischemia-reperfusion of testis stimulates an intracellular signaling cascade in the endothelial cells that results in neutrophil recruitment, an increase in intracellular reactive oxygen species (ROS), and eventual germ cell-specific apoptosis (3-5).

Cannabinoids have a long history of consumption for recreational and medical reasons (6). Endogenous

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cannabinoids first identified in the central nervous system (7). They have several physiological roles in central nervous system and peripheral tissues (8), and act through interaction with G protein-coupled membrane receptors, namely CB1 and CB2 receptors, which are present throughout the body (7). The presence of a cannabinoid receptor in testes (9) suggests a physiological role for cannabinoids in testicular tissue.

Anandamide or N-arachidonylethanolamine is an endogenous ligand for cannabinoid receptors that was discovered in porcine brain. It is a modified form of arachidonic acid (10). Anandamide can serve to protect the brain against neuronal injury (11, 12) and it has endothelial protective effect against ischemic preconditioning in rat coronary arteries. So, this study was designed to evaluate whether anandamide treatment reduces I/R injuries in testes (13).

MATERIALS AND METHODS

Drugs

Drugs were purchased from Tocris (U.K). Anandamide (Cat. No 1339) was dissolved in phosphate buffer solution (PBS), tween and ethanol (9:0.5:0.5) and AM251 (Cat. No 1117) was dissolved in dimethyl sulfoxide (DMSO).

Animals

Study groups consisted of 36 adult male Sprague-Dawley rats weighting 240 to 260 gm. All animals were treated humanly and in compliance with the recommendation of the animal care committee of university and the principles of laboratory animal care (NIH publication No. 85-23, revised 1985). The rats were housed in temperature- controlled room (24 ± 1 °C) on 12 h light – 12 h dark cycle with free access to food and water.

The rats were divided into 6 groups of 6 each. Surgical procedure was done under general anaesthesia induced by intraperitoneal one shot injection of ketamine HCl (50 mg/kg) and chlorpromazine (25 mg/kg). The skin of scrotal area was shaved and then prepared with 10 % povidone iodine solution. A midscrotal vertical incision was performed to get access to both testes. Torsion was created by twisting the right testes 720° in counter clockwise direction and maintained by fixing the testes to scrotum with a 6/0 nylon suture passing through the tunica albuginea and dartos. After 1 hour of ischemia the suture was removed and right testes was detorted and replaced into scrotum for 4 hour of reperfusion. During sham operation the right testes was brought through the incision and then replaced without twisting, and a nylon suture was placed through the tunica albuginea. After each surgical intervention the incision was closed. At the end of experiment the rats were sacrificed with overdose of sodium pentobarbital, and bilateral orchiectomies were performed for histological examinations and measurement of tissue malondialdehyde (MDA) level and catalase activity.

Groups

In base line group (normal group), bilateral orchiectomies performed after anaesthesia. In torsion- detorsion (T/D) group, after 1 hour testicular torsion and 4 hour detorsion, bilateral orchiectomies were performed. In anandamide group, half an hour before torsion, anandamide (10 mg/kg) was injected intraperitonally. Both testes were harvested 4 hours after detorsion. In AM251 group, the same procedure was done as in the T/D group but AM251 (0.5 mg/kg) was administered 45 min before torsion. In anandamide –AM251 (An/AM) group, the same surgical procedure was done as in the T/D group, but AM251 was injected 45 min before torsion, and then anandamide was administered 30 min before torsion. In control group, the organs were harvested after sham operation.

Malondialdehyde (MDA) assay

MDA is an end product of peroxidative decomposition of polyenic fatty acids in the lipid peroxidation process and its accumulation in tissues is indicative of the extent of lipid peroxidation. Tissue MDA was measured using the thiobarbituric acid reactive substance assay, as described by Ohkawa *et al.* (14). In brief, testis tissues were homogenized in 1.15% KCl to make a 10 % (w/v) homogenate. To 0.1 ml of tissue homogenates, were added 0.9 ml of 1.8 % sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution (pH 3.5) and 1.5 ml of aqueous solution of thiobarbituric acid (TBA). The mixtures were heated at 95°C for 60

min. After cooling with tap water, 5 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. The organic layer was taken and its absorbance at 532 nm was measured. 1,1,3,3-tetramethoxypropane (TMP) was used as an external standard and the level of lipid peroxides was expressed as nmol of MDA per gram wet tissue.

Catalase activity

Tissue catalase activity was measured according to method of Aebi (15). Tissue sections were homogenized in triton X-100 (1%) and the homogenates were diluted with potassium phosphate buffer. The reaction was initiated by the addition of Hydrogen peroxide (H_2O_2) to reaction mixture and the level of enzyme activity was quantitated according to ability of tissue catalase to decompensate H_2O_2 by monitoring of decrease in absorbance at 240 nm. The value of log A1/A2 for a measured time interval was used for unit definition due to the first-order reaction of enzyme.

Histopathology

All testes were immersed in Bouin's fixative and kept at 4 C° for 5 days, and then they were embedded in paraffin. Five µm sections were obtained deparaffinized and stained with hematoxylin and eosin. Three slides prepared from upper, lower, and mid portions of testes were evaluated by two pathologists in a blind, randomly numbered fashion. A 4-level grading scale similar to that of Cosentino *et al.* was used to quantify histologic injury (16). Grade 1 showed normal testicular architecture with an orderly arrangement of germinal cells. Grade 2 injuries showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules. Grade 3 injury exhibited disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders. Grade 4 injury defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells.

Statistical analysis

Data are expressed as the mean ± SD. ANOVA was used for statistical analysis of data among all groups. Multiple comparisons were made using Tuckey's procedure with $P < 0.05$ considered statistically significant.

RESULTS

Figure 1 shows ipsilateral testicular MDA level in figure 2 has shown ipsilateral catalase activity of all groups. The ipsilateral values of MDA and catalase activity of control group did not show significant changes compared with normal group. Ipsilateral MDA levels were significantly increased in T/D group versus control group ($P < 0.001$). Ipsilateral MDA levels in anandamide group were significantly lower than T/D group ($P < 0.01$), while MDA levels in anandamide group were not significantly different from control group.

There were significant decreases in catalase activity in T/D group versus normal and control groups ($P < 0.01$). This value in anandamide group was also significantly higher than T/D group ($P < 0.05$). It was also shown that MDA levels in An/AM group were significantly higher than anandamide group ($P < 0.001$). Furthermore, in An/AM group, catalase activity was significantly lower than that of anandamide group ($P < 0.01$). MDA level and catalase activity of contralateral testes of all groups have shown in figure 3 and figure 4. The values of these two parameters in the contralateral testes of torsion experienced groups were not significantly different from normal and control groups. Table 1 shows histopathologic results of ipsilateral testes in all groups. The ipsilateral testes in normal group and sham group demonstrated grade 1 and they did not undergo any morphologic changes (Fig. 5). Moreover, the ipsilateral testes of all groups, which experienced testicular torsion, showed similar histopathologic changes and these groups demonstrated grade 2 injuries (Fig. 6). The contralateral testes in all groups showed normal testicular architecture (grade 1).

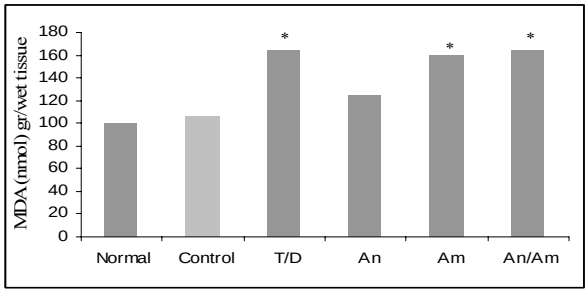

Fig. 1. MDA level and catalase activity in the ipsilateral testes. Data are given as mean ± SD. *, significantly different compared to control group ($P < 0.001$). MDA, malondialdehyde; T/D, torsion/detorsion; An/AM, anandamide/ AM251.

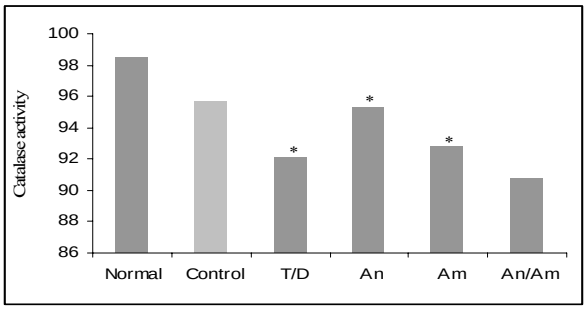

Fig. 2. MDA level and catalase activity in the contralateral testes. Data are given as mean ± SD. ** Significantly different compared to control group ($P < 0.01$). Abbreviations: MDA, malondialdehyde; T/D, torsion/ detorsion; An/AM, anandamide/AM251.

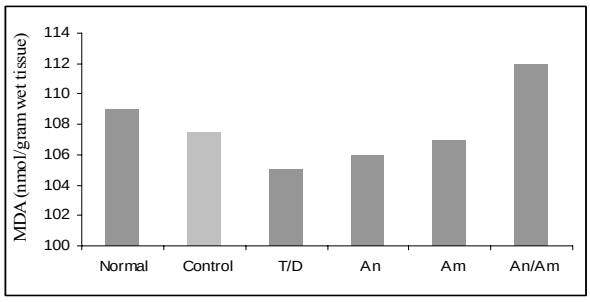

Fig. 3. MDA level in the contralateral testes. Data are given as mean ± SD. There are not any significant changes between groups. Abbreviations: MDA, malondialdehyde; T/D, torsion/detorsion; An/AM, anandamide/ AM251.

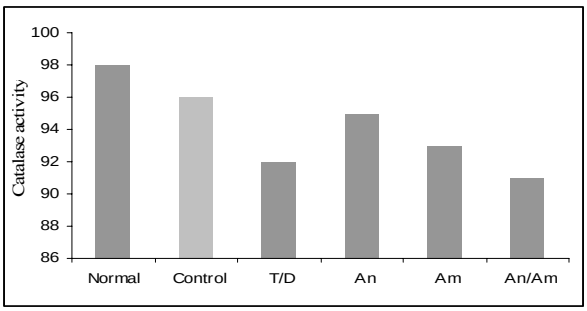

Fig. 4. Catalase activity in the contralateral testes. Data are given as mean ±SD. There are not any significant changes between groups. Abbreviations: MDA, malondialdehyde; T/D, torsion/detorsion; An/AM, anandamide/ AM251.

Table 1. Histologic evaluation of ipsilateral testes*

Group	Grade 1	Grade 2	Grade 3
Normal	6	0	0
Control	6	0	0
T/D	0	6	0
Anandamide	0	6	0
AM251	0	6	0
An/AM	0	6	0

* Data are given as number.
Abbreviations: T/D, torsion/detorsion; An/AM, anandamide/ AM251.

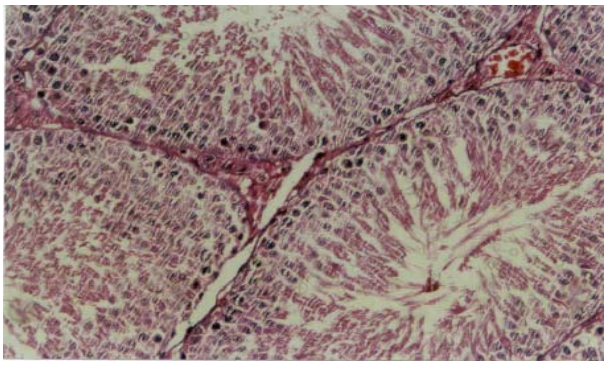

Fig. 5. Grade 1, normal testicular architecture with an orderly arrangement of germinal cells (H&E staining).

DISCUSSION

Cannabinoids treatment has been used successfully to decrease ischemia-reperfusion (IR) injuries in heart (7, 13) and brain (11, 12, 17). These successes had led us to attempt such treatment with a rat model of testicular torsion. In this study, IR caused an increase in MDA level (the indicator of ROS level) of ipsilateral testis in T/D group. ROS or Reactive Oxygen Species is produced during reperfusion component of IR. There are two sources of free radicals (ROS) in post-ischemic tissues. One source of ROS is the hypoxanthine-xanthine oxidase reaction (18). Ischemia causes an increase in intracellular hypoxanthine as a result of ATP breakdown and then, during reperfusion, xanthine oxidase converts hypoxanthine to uric acid plus large quantities of superoxide radicals in the presence of oxygen. Neutrophil recruitment is the other source of ROS production (4). The increase in pro-inflammatory cytokines (TNF-α, IL-1β, and IL-8) after IR of the testis is correlated with activation of

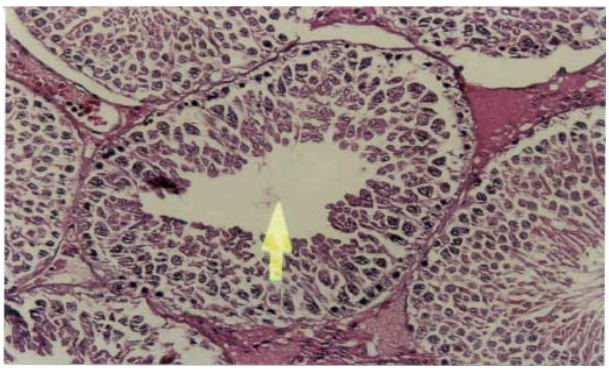

Fig. 6. Grade 2, injuries showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules (H&E staining).

signaling pathway leading to expression of E-selectin in vascular endothelial cells (5). E-selectin aids in the capture and attachment of blood neutrophils to the vascular endothelium. Other cellular adhesion molecules, such as integrins, then aid in the firm adhesion and transmigration of neutrophils (4). Such cascades occur in other tissues after IR (19, 20). Neutrophils recruited to vascular endothelium of testis may produce excess amounts of cytokines (21), which results in the recruitment of more neutrophils and further expression of E-selectin. Neutrophils transmigrate through the endothelium into the interstitium of the testis and then release factors such as ROS that may directly cause apoptosis in germ cells (4).

In the other hand, the superoxide radicals, produced during reperfusion, rapidly react with nitric oxide (NO) molecules, produced during ischemia (22, 23), and form peroxynitrite which promotes further damage to reperfused tissues (24).

The administration of anandamide resulted in a decrease in the level of MDA in anandamide group compared to T/D group. It has been reported previously that the administration of cannabinoids reduces the production of pro-inflammatory cytokines (25). So, anandamide might reduce the production of E-selectin through reducing the release of pro-inflammatory cytokines after IR and thus inhibit neutrophil recruitment, leading to a decrease in ROS level. The anti-oxidative effect of cannabinoids also leads to a decrease in NO production by inhibition of the redox-sensitive nuclear factor-κB activation, which is required for the expression of inducible NO-producing enzyme; NO synthase (26, 27). We also detected a significant decrease in catalase activity in the T/D group compared to control group, which would be translated to decompensate anti-oxidant power of testicular tissue. Administration of anandamide inhibited the reduction of catalase activity after IR in anandamid-treated group. It is in harmony with anti-oxidative effect of cannabinoids described earlier. The data also suggested that the effects of anandamide were mediated via cannabinoid receptors, since AM251, a cannabinoid antagonist, could abolish these effects.

The effect of unilateral torsion on the contralateral testes is controversial. It has been demonstrated that ipsilateral torsion does not result in contralateral testicular damage in rats (28, 29). Similarly in our study, though 1 hour of torsion followed by 4 hours of detorsion resulted in significant changes in histopathological and biochemical analysis of the ipsilateral testes, our results did not reveal any changes in the contralateral testes.

Conflict of interests

We have no conflict of interests.

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