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ORIGINAL ARTICLE

Prophylactic Effects of Verapamil in Testicular Ischemia-reperfusion Damage in Rats

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Abstract

Introduction: Testicular torsion, migration of neutrophils to the ischemic region and the formation of free oxygen radicals are important factors in the occurrence of ischemia-reperfusion damage in testes. Verapamil HCl is a phenylalkylamine derivative of the L-type voltage-dependent calcium channel blocker. In our study, we evaluated the prophylactic effects of the Verapamil in testicular ischemia-reperfusion damage.

Methods: Twenty-one, eight weeks old adult, male Wistar-Albino rats were randomly divided into three groups as follows: Sham, I/R, I/R+Verapamil HCl. In Sham and I/R groups, saline was injected intraperitoneally at the second hour; in I/R+Verapamil HCl group, Verapamil was injected intraperitoneal at the second hour. Spermatogenic functions were evaluated according to Johnsen criteria, and then, average scores were calculated. $P<0.05$ was statistically significant.

Results: We compared all histopathological and molecular parameters derived from all groups. Average levels of inflammation mediators (TNF-α and IL-1β) were calculated. In the early period, compared with the Sham group, TNF-α levels increased statistically significant in thel/R group, which did not receive any medical treatment. Also, we saw that Verapamil HCl treatment avoided an increase in TNF-α level and provided the same levels as the Sham group. Glutathione peroxidase (GPx), which is antioxidant in tissue levels, significantly decreased in thel/R group, which did not receive Verapamil HCl as compared with the Sham group. Verapamil HCl avoided this situation. Although GPx expression scores were higher in the I/R group than the Sham group, Verapamil HCl treatment takes these scores to the same levels as in the Sham group. For spermatogenesis, there was no statistically significant difference between groups according to Johnsen scoring system.

Discussion and Conclusion: I/R generated by T/D, in the early period, causes an increase of inflammation mediators in blood. It resulted in an increase in GPx activity, which decreased anti- oxidant capacity in tissue, and it has no effect to spermatogenesis parameters in four hours period. Verapamil HCl reduces damage in testicular I/R.

Keywords: Ischemia-reperfusion; rat; testes; verapamil.

Testicular torsion and torsion of the spermatic cord are urologic emergencies in newborns, child and adolescence. Incidence is approximately 1/4000 until 25 years old [1]. The rate of survival of testis detorsioned by surgery is approximately 48-88% [2]. However, the spermatogenic function is not known in these testes. It is stated that simple orchiectomy must be done in late cases and also in these cases may cause loss of function in contralateral and infertility [3]. Although when irreversible changes in testis after torsion begin is not clear, it is recommended that in-

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tervention or surgery should be carried out in 4-6 hours [4].

The testis is sensitive to the damage of free oxygen radicals. Germinal cells are seriously damaged in oxidative stress [5]. In studies in 38% of the males who have a history of torsion, sperm count is less than 20 million per ml [6]. NO released from tissue and TNF-α, IL1-B released from neutrophils increase inflammation. Neutrophils play an important role in the occurrence of ROS by interacting with the oxidative and nitrosative system. For level and occurrence of free oxygen radicals in tissue enzymes, such as GPx, SOD, catalase and iNOS, which take place in these systems are calculated [7].

Tissue damage as a result of hypooxygenation in torsion can be reversed by immediate detorsion. In addition, protective agents can provide more function in testis after detorsion against reperfusion damage after surgery. Thus, many protective agents against reperfusion damage are investigated.

Materials and Methods

The experimental protocol was approved by the Marmara Experimental Animal Center (DEHAMER) Ethics Committee. Twenty-one, an adult male weighing 250-350 g at the 8-week Wistar-Albino rats were used in our study. Before the experiment in two weeks, habituated subjects to laboratory conditions (22 °C ± 2, 12 h light/12 h dark, one atmospheric pressure) were housed in cages dry. In diet, the standard rat chow (Optima brand) and city water were used. Before the induction of anesthesia before the procedure, rats were weighed on 0.01 g precision scales. Weights were recorded. After completion of all procedures, including operation, the subjects, while under general anesthesia, were sacrificed using decapitation.

Verapamil HCl was used 5-20 mg/kg dose in adults. The dose of Verapamil HCl administered to the rats was determined as 10 mg/kg. For anesthesia, 100 mg/kg Ketamine and 5 mg/kg Xylazine intraperitoneally (ip) were administered. During operation, sterile conditions were provided for each rat.

Rats were divided into three groups as follows: Sham, IR, IR + verapamil. In all three groups, after the anesthesia operation of rats, the left testis was separated from the gubernacular structure by mid-scrotal incision. In the Sham group, in two hours, intraperitoneally 10 mg/kg of saline (SF) without giving any further intervention was injected than by 5-0 prolene suture testis was fixed to the scrotum. Venous blood samples were taken in the six-hour, then, rats were sacrificed, and bilateral orchiectomy was performed.

IR and IR + verapamil group, the testis and cord elements were torsioned by rotating counter-clockwise 720 degrees. After two hours, torsion time testis was detorsioned, then, intraperitoneal 10 mg/kg of saline was administered to the IR group, 10 mg/kg of verapamil HCl to IR + verapamil group and finally by giving any further intervention by 5-0 prolene suture testis was fixed to the scrotum. After the 4-hour reperfusion period, the venous blood sample was taken, subjects were sacrificed, then underwent bilateral orchiectomy.

TNF-α and IL-1ß levels in venous blood taken into tubes containing EDTA were measured. Histological evaluation was made for the tissue damage. For the evaluation of spermatogenic function, each testis was scored according to the Johnsen criteria. In tissue, glutathione peroxidase (GPx) levels were determined by immunohistochemical methods.

SPSS v.15.0 program was used for statistical evaluation. Obtained all numerical, nominal and ordinal data were compared using the Kruskal-Wallis test, Mann-Whitney U test and Dunn's test. $P<0.05$ was considered statistically significant.

Results

The average weight of 21 Wistar albino rats included in this study was 328.95±42.13 g. These averages were similar in all three groups; there was no statistically significant difference between them. The average weight of orchiectomy specimens extracted from mouse testis was similar between groups. The mean weight of the testes was 1.53, 1.63 and 1.65 in sham, I/R and I/R + verapamil group, respectively (Table 1).

Table 1. Evaluation of the parameters for each group

	n	Rat weight (average±SD) (gr)	Testis weight (average±SD) (gr)	Tubulus diameter (µm)
Sham	7	326.14±28.86	1.53±0.06	313.37±32.57
I/R	7	313.57±52.06	1.63±0.14	289.01±20.75
I/R + V	7	347.14±23.34	1.65±0.11	258.40±16.51

SD: standard deviation; I/R: ischemia-reperfusion; V: verapamil; gr: gram; µm: micrometer.

When TNF-αand IL-1ß levels were evaluated, two hours four hours after ischemia-reperfusion for I/R group compared to the sham group. Statistically significantly higher levels of TNF-α were found (p=0.021). We determined that verapamil HCl treatment was administered during reperfusion (I/R + V, group 3) decrease levels of TNF-α statistically significant compared to untreated I/R group (group 2) (p=0.015). In addition, in the group treated with verapamil HCl treatment when compared to the sham group, TNF-α levels were observed to be similar (p=0.74). When IL-1ß levels of all groups were compared, there was no statistically significant difference (p=0.55) (Fig. 1).

When we compare extent, intensity, levels of expression score of GPx, we found that GPx of positively stained cells during reperfusion in the group administered verapamil (I/R + V) the ischemia-reperfusion (I/R) compared with less dye was found to be statistically significant (p<0.05). Also, in I/R+V group, similar levels of GPx scores were found with the sham group (p<0.05). Considering the prevalence of GPx scores between groups, there were not any statistically significant differences (p>0.05). When GPx expression scores evaluated, the mean score of I/R group compared to the sham group was found to be significantly higher. 30 min before reperfusion of the verapamil treatment significantly lowered the levels of GPx (p<0.05) (Table 2).

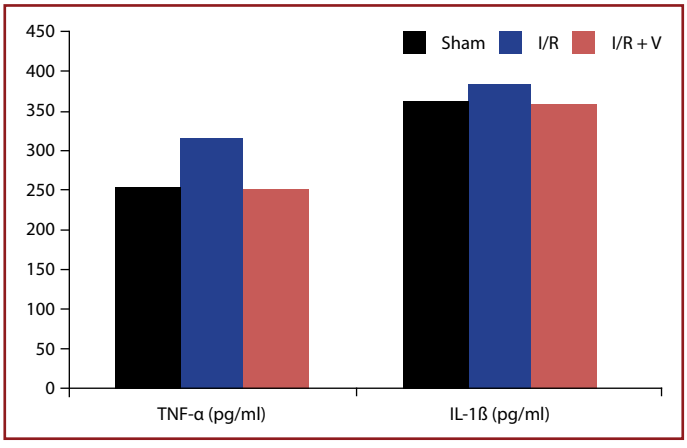

Figure 1. Comparison of blood TNF-α and IL-1β.

I/R: ischemia-reperfursion; V: verapamil; TNF- α: tumor necrosis factor alpha; IL-1β: interleukin 1 beta.

The mean Johnsen scores compared; in the group treated with the sham operation, in both testes, seminiferous tubules and interstitium were normal. In the I/R group, compared to the sham group, Johnsen score did not change significantly. When the I/R and sham group compared, verapamil HCl treatment did not affect spermatogenesis testicular parameters. It does not cause any increase (p>0.05).

Discussion

Testicular torsion affecting adolescents and young men is a urologic surgical emergency. The incidence is reported as 1/4000 up to age 25 [8]. When the patient is referred to the emergency department, immediate detorsion is necessary to prevent damage to the testicles. However, despite a successful operation, testicular atrophy may develop over time, and this may cause infertility. This case arises from the degradation of the testicle blood and reperfusion-induced oxidative stress after detorsion operations [9]. Therefore, testicular torsion been urgently be detorsioned, identification of the treatment to protect testis from I/R injury to recover testis and to prevent the oxidative stress damage would be useful. Ischemia-reperfusion damaged the testicular cells membrane. Membrane injury leads to calcium (Ca^2+) involvement from the intercellular distance into the cell [10]. Calcium is an important second messenger involved in intra- and extracellular signaling cascades and plays an essential role in cell life and death decisions [11]. Calcium ions stimulate apoptosis by the initiation of the apoptotic cascade [12]. With the use of the calcium channel blocker (e.g., verapamil), providing the intracellular calcium homeostasis, tissue could be protected against ischemia-reperfusion damage and apoptosis. In our study, performed on rats in an animal model, the Verapamil

Table 2. Comparison of the GPx expression score averages

	n	Extent (average±SD)	Intensity (average±SD)	Expression score (average±SD)
Sham (ipsilateral)	7	1.42±0.53	1.86±0.38	2.57±0.38
Sham (contralateral)	7	1.28±0.48	1.57±0.53	2.14±1.35
I/R (ipsilateral)	7	1.86±0.38	1.86±0.90	3.43±1.90
I/R (contralateral)	7	1.43±0.53	1.57±0.53	2.29±1.25
I/R + V (ipsilateral)	7	1.29±0.49	1.86±0.38	2.42±1.13
I/R + V (contralateral)	7	1.14±0.38	1.86±0.38	2.14±0.90

GPx: glutation peroxidase; SD: standard deviation; I/R: ischemia-reperfusion; V: verapamil; gr: gram; p<0.05; Sham-I/R and I/R-I/R+V groups (ipsilateral); p>0.05, Sham-I/R, Sham-I/R+V and I/R-I/R+V groups (contralateral).

protection against reperfusion injury in torsion and detorsion of the testis were investigated.

Detorsioned testes after the operation are very sensitive to free radical damage. Several therapeutic agents recommended in the literature can be used as an antioxidant to prevent damage, but they are still under intensive research. I/R studies conducted with many drugs, such as Trimetazidine [13], erythropoietin [14], taurine [15], curcumin [16], sildenafil [17], montelukast [18], lycopene [19], ginkgo biloba [20], show that prior administration of reperfüzyo antioxidant agents reduce apoptosis by reducing the amount of free oxygen radicals in ischemic testicular cell damage. However, because these treatments are still in the experimental stage, having many of the side effects and problems about providing in emergency conditions, they are still not used daily practice [17]. When our knowledge about the oxidative and nitrosative system increases, the preventability of I/R injury developing after T/D in testicular tissue will also increase. In this sense, antioxidants and effectiveness of the use of agents reducing apoptosis and increasing regeneration should be investigated. To identify active agents, animal models are being studied more than human studies due to ethical problems.

I/R injury mechanism, consisting of ROS to cause neutrophil activation and adhesion, and thus is suggested to lead to tissue. The first stage in the formation of damage is neutrophil activation. To support that in studies conducted with serum with anti-neutrophil to prevent reperfusion injury or monoclonal antibodies against leukocyte adhesion molecules show that neutrophils are responsible for the increase in microvascular permeability during reperfusion. Thus, neutrophils have an important role in the increase in microvascular permeability during the I/R injury and subsequent pathological changes [9].

The effects of verapamil on testis and semen parameters have been evaluated in several experimental studies. In the study conducted by Gao et al., [21] in a rat model made torsion-detorsion, before detorsion, verapamil and hypothermia have been shown to have positive effects on spermatogenesis. Co-administration also has been reported to be much more effective. In a study published in 2010 conducted by Shirazi et al., [22] in rats undergoing torsion-detorsion a week of regular systemic administration of verapamil had no effect on the morphology in semen analysis, however, has been reported to provide a positive impact on the number and mobility. In our study, in rats undergoing torsion-detorsion, before detorsion, systemic administration of verapamil do not affect Johnsen scores for spermatogenesis ratings, increase tissue levels of GPx activity and decrease blood levels of TGF α.

Glutathione peroxidase (GPx) is the generic name of an enzyme family that protects the organism from oxidative damage. The biochemical function of GPx is to reduce the level of hydrogen peroxide, lipid peroxides and the corresponding alcohols. Isozymes encoded by separate genes vary in a wide variety of tissues and cells [23].

To evaluate in our study, testis tissue from subjects sacrificed by decapitation after orchiectomy were analyzed immunohistochemically. According to this observation, when we analyze expression scores, we see that I/R and GPx activity increase significantly according to sham groups and verapamil HCl treatment 30 minutes before reperfusion decrease the GPx levels similar to the sham group. This means a reduction of oxygen radicals in tissue and further to minimize tissue damage. However, after the torsion-detorsion and I/R, its rapidly increasing amount of tissue and by combining with oxygen to create peroxynitrite causes DNA damage and apoptosis. In many experimental studies performed, increase of testicular tissue GPx after reperfusion has been shown, and this has been associated with tissue damage [24–26].

There is no common view on experimental models of torsion when and what extent spermatogenesis is affected after reperfusion. In the studies according to Johnsen criteria, with detorsion and after reperfusion ranging from three months to two hours, spermatogenic function significantly worsened was shown [13, 16]. In a study conducted by Turner et al., [27] how reperfusion affected sperm motility and sperm concentration in the different duration were evaluated. According to this study, there was no significant change according to the control group on day 7 of reperfusion, whereas only 30 and 60 days, a significant decrease in these parameters was observed. In our study, spermatogenic function in both testes was evaluated according to the criteria of the Johnsen. In this evaluation, the spermatogenic functions were assessed in both testes of subjects sacrificed in four hours after reperfusion, but similar results were obtained in the comparison of all groups and both testes. Considering the different results in the literature, this result is an acceptable result because many factors, such as torsion shape, degree, direction, duration of torsion and detorsion, are likely to affect results.

Conclusion

In this study, evaluating the effects of verapamil HCl therapy in the I/R model generated by T/D in unilateral testicular the relationship between the groups were compared regarding histopathologic and molecular parameters, and

the following conclusions were reached:

T/D induced I/R leads to an increase in inflammatory mediators in blood. An increase in GPx activity has resulted. The antioxidant capacity was determined in tissue. Spermatogenesis parameters are not affected in the 4-hour

Corruption in biochemical and histopathological parameters as a result of all this is reversed with the treatment of verapamil HCl. In other words, verapamil HCl reduces the damage induced by the I/R in testis. The data of this experimental study suggest that the usability of treatment with verapamil HCl in testicular torsion should be investigated.

Ethics Committee Approval: Marmara Experimental Animal Center (DEHAMER), 2013.

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