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Reperfusion injury after detorsion of unilateral testicular torsion

Feza M. Akgür[1], **Kamer Kılınç**[2], **Tanju Aktuğ**[1]

[1] Department of Pediatric Surgery, Dokuz Eylül University, Medical Faculty, İzmir, Turkey
[2] Department of Biochemistry, Hacettepe University, Medical Faculty, Ankara, Turkey

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Summary. Reperfusion injury has been well documented in organs other than testis. An experimental study was conducted to investigate reperfusion injury in testes via the biochemical changes after unilateral testicular torsion and detorsion. As unilateral testicular torsion and varicocele have been shown to affect contralateral testicular blood flow, reperfusion injury was studied in both testes. Given that testicular blood flow does not return after 720° testicular torsion lasting more than 3 h, the present study was conducted after 1 and 2 h of 720° torsion. Adult male albino rats were divided into seven groups each containing ten rats. One group served to determine the basal values of biochemical parameters, two groups were subjected to 1 and 2 h of unilateral testicular torsion respectively, two groups were subjected to detorsion following 1 and 2 h of torsion respectively, and two groups underwent sham operations as a control. Levels of lactic acid, hypoxanthine and lipid peroxidation products were determined in testicular tissues. Values of these three parameters obtained from the sham operation control groups did not differ significantly from basal values ($P > 0.05$). All three parameters were increased significantly in both ipsilateral and contralateral testes after unilateral testicular torsion when compared with basal values ($P < 0.01$ and $P < 0.05$, respectively). Detorsion caused significant changes in lipid peroxidation products levels in ipsilateral but not in contralateral testes when compared with values obtained after torsion ($P < 0.01$ and $P > 0.05$, respectively). It is concluded that ipsilateral testicular torsion causes a decrease in perfusion not only in the ipsilateral but also in the contralateral testis. Additionally, detorsion following up to 2 h of 720° torsion causes reperfusion injury in ipsilateral but not in contralateral testis.

Key words: Oxygen free radicals – Reperfusion injury – Testis torsion

Although there are some findings to the contrary [19] the contralateral testis may be damaged after unilateral testicular torsion. Despite several hypotheses the mechanism of contralateral damage remains unclear. Electromagnetic and radioisotopic blood flow measurements have shown that unilateral testicular torsion causes a decrease in contralateral testicular blood flow [13, 17] which gradually increases after detorsion [17]. It has therefore been proposed that the decrease in contralateral testicular blood flow may be the cause of contralateral testicular damage [13, 17]. A decrease in blood flow is known to cause hypoxia, which results in elevated levels of lactic acid, hypoxanthine [16] and lipid peroxides [11] in the tissues. Unilateral 720° testicular torsion has been shown to cause an increase in all these parameters in both ipsilateral and contralateral testis [1, 2]. Additionally, the gradual increase following a decrease in blood flow may lead to further tissue damage through increased lipid peroxidation following production of oxygen-derived free radicals, as shown in other organs [6, 11, 15]. This phenomenon is called "post-hypoxic reoxygenation injury" or, more commonly, "reperfusion injury" [6, 11, 15].

In our previous study we investigated reperfusion injury via the biochemical changes in both testes after detorsion of 720° testicular torsion lasting more than 6 h. We were unable to find evidence of reperfusion injury and postulated that hypoperfusion may persist in both testes [1]. This is consistent with the results of other studies. Turner et al. [19] showed that ipsilateral testicular blood flow does not return after testicular torsion lasting more than 4 h; however it does return after 1 or 2 h of torsion [19] and yet significant histopathologic changes take place both in ipsilateral and contralateral testes [4, 5, 19].

An experimental study was conducted to investigate reperfusion injury in both testes via the biochemical changes after detorsion of unilateral testicular torsion lasting 1 or 2 h.

Correspondence to: Feza M. Akgür, Dokuz Eylül Universitesi, Tıp Fakültesi, Çocuk Cerrahisi Anabilim Dalı, İnciraltı 35340 İzmir, Turkey

Materials and methods

Seventy adult male albino rats were divided into seven groups each comprising ten rats. All surgical procedures were performed with the rats under intraperitoneal one-shot ketamine (30 mg/kg) anesthesia. Torsion, detorsion and sham operations were all performed on the left testes through standard ilio-inguinal incisions. Torsions were created by rotating the left testes 720° in a clockwise direction. Torsions were maintained by fixing the testes to the scrotum by a silk suture through the tunica albuginea. During sham operations the testes were brought through the incisions and replaced and a silk suture placed through the tunica albuginea. After each surgical intervention the incisions were closed and the rats left free in their cages. At the end of the experiments bilateral orchiectomies and right nephrectomies were performed.

Group 1 (basal value group): After anesthesia the aforementioned organs were harvested to determine basal values of biochemical parameters in these organs.

Groups 2 and 3 (torsion groups): The aforementioned organs were harvested at the end of 1 or 2 h of unilateral testicular torsion respectively.

Groups 4 and 5 (detorsion groups): Detorsion was carried out after 1 or 2 h of unilateral testicular torsion respectively. Aforementioned organs were harvested 2 h after detorsion in both groups. Thus testes were reperfused for 2 h. A reperfusion period of 2 h was selected on the basis that lipid peroxides appear a few minutes after reperfusion and are retained for as long as 12 h [22].

Group 6 (one sham operation control group): A sham operation was performed and the aforementioned organs harvested 1 h later.

Group 7 (two sham operations control group): The sham operation was repeated 2 h after the first sham operation. The aforementioned organs were harvested 2 h after the second operation.

All organs were placed in glass bottles with rubber caps, labeled and stored in a deep freeze. Lactic acid, hypoxanthine and thiobarbituric acid reactive products (TBAR) of lipid peroxidation were determined in the tissues. ANOVA was used for statistical analysis of the data among groups. Multiple comparisons were made using Tukey's procedure. Comparisons between right and left testicular data were made by *t*-tests.

Biochemical determinations

Tissues were homogenized in 150 mM ice-cold KCl to make a 10% homogenate, using a glass Teflon homogenizer. Immediately afterwards 2 ml of this homogenate was pipetted into a tube containing 2 ml cold 8% (v/v) $HClO_4$. The mixture was shaken vigorously and kept cold until centrifuged. The supernatant fraction (deproteinization supernatant was kept frozen until the determinations of lactate and hypoxanthine concentrations were performed.

Determination of lipid peroxides

Lipid peroxides in tissues were determined by the method of Uchiama and Mihara [21]. Three milliliters 1% phosphoric acid and 1 ml 0.6% thiobarbituric acid (TBA) solution were added to 0.5 ml 10% tissue homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After cooling, the color was extracted into 4 ml *n*-butanol and the absorbance was measured. The amounts of lipid peroxides were calculated as thiobarbituric acid reactive (TBAR) substances ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Determination of lactic acid

The concentrations of lactate in tissues were determined enzymatically using LDH and NAD^+ [16]. Lithium lactate was used as the lactate standard. The absorbances of blank, standards and deproteinization supernatants prepared and studied under identical conditions were measured at 340 nm. Comparing the absorbance of samples with lactate standards, tissue lactate levels were calculated as micromoles lactate per gram of wet tissue.

Determination of hypoxanthine

For the determination of hypoxanthine 0.1 ml deproteinization supernatant was neutralized with 0.5 ml K_3PO_4 (0.7 M). The tubes were kept at 0°C for 5 min and precipitated $KClO_4$ was removed by centrifugation [10]. A 0.5 ml aliquot of clear supernatant was mixed with 2 ml 0.1 M TRIS-HCl buffer (pH 7.4) containing 3 mM EDTA. The absorbance of the resulting mixture at 37°C was read at 293 nm. Then 0.04 units of xanthine oxidase was added in a volume of 20 µl (the absorbance contributed by the enzyme was 0.200). Tubes were incubated at 37°C for 10 min and the absorbances read again at 293 nm. The conversion of hypoxanthine to uric acid was observed to be complete in 5–6 min. The absorbance changes were used to calculate the hypoxanthine concentration taking the molar absorptivity of uric acid to be 12200 $M^{-1}\ cm^{-1}$ [10, 18]. Hypoxanthine concentrations in tissues were also determined using hypoxanthine as standard. Standards were prepared and studied under the same conditions. The two methods gave identical results. The hypoxanthine levels in tissues are given as nanomoles per gram of wet tissue.

Results

Basal intratesticular and intrarenal lactic acid, hypoxanthine and TBAR values are shown in Tables 1–3.

The values of these three parameters obtained from the organs of animals in the sham operation control groups did not differ significantly from basal values ($P > 0.05$) (Tables 1–3).

After unilateral testicular torsion, lactic acid, hypoxanthine and TBAR values increased significantly compared with basal values in both ipsilateral and contralateral testes ($P < 0.01$ and $P < 0.05$, respectively) but not in kidneys ($P > 0.05$) (Tables 1–3).

Compared with levels obtained after 1 or 2 h of torsion, detorsion caused a significant decrease in lactic acid and hypoxanthine and an increase in TBAR values in ipsilateral testes ($P < 0.01$) but did not affect any of the three parameters in contralateral testes and kidneys ($P > 0.05$). However, most of the values obtained after detorsion were significantly increased compared with basal values in both ipsilateral and contralateral testes ($P < 0.05$) but not in kidneys ($P > 0.05$) (Tables 1–3).

In the torsion and detorsion groups, all three parameters obtained from contralateral testes were significantly lower than those obtained from ipsilateral testes ($P < 0.05$).

Discussion

Hypoxia results in elevated levels of lactic acid, hypoxanthine [16] and lipid peroxides [11] in tissues. Sham operation control groups showed that anesthesia and

Table 1. Lactic acid, hypoxanthine and thiobarbituric acid reactive (TBAR) substances values in ipsilateral testes (mean ± 1 SD)

Groups	Lactic acid (μmol/g wet tissue)	Hypoxanthine (nmol/g wet tissue)	TBAR (nmol/g wet tissue)
Basal	3.51 ± 0.69	27.59 ± 2.05	84.69 ± 7.29
1 h torsion	7.79 ± 0.87[a]	429.19 ± 28.40[a]	129.87 ± 12.76
2 h torsion	10.29 ± 1.06[a,b]	423.97 ± 69.84[a]	375.98 ± 71.91[a,b]
2 h after detorsion of torsion for 1 h	7.44 ± 0.81	352.36 ± 47.33[a,c]	417.55 ± 61.08[a,c]
2 h after detorsion of torsion for 2 h	8.79 ± 1.27[a,c]	292.79 ± 37.92[a,c]	502.73 ± 85.33[a,c]
One sham operation	3.89 ± 0.73	31.32 ± 2.78	79.66 ± 8.52
Two sham operations	4.39 ± 0.59	34.25 ± 3.09	76.03 ± 9.93

[a] $P < 0.01$ compared with basal value group
[b] $P < 0.01$ compared with previous torsion group of shorter duration
[c] $P < 0.01$ compared with predetorsion level

Table 2. Lactic acid, hypoxanthine and thiobarbituric acid reactive (TBAR) substances values in contralateral testes (mean ± 1 SD)

Groups	Lactic acid (μmol/g wet tissue)	Hypoxanthine (nmol/g wet tissue)	TBAR (nmol/g wet tissue)
Basal	3.48 ± 0.58	27.60 ± 1.55	80.72 ± 4.54
1 h torsion	3.88 ± 0.16	37.27 ± 4.87[a]	85.38 ± 3.78
2 h torsion	4.15 ± 0.43[a]	38.66 ± 7.71[a]	88.88 ± 4.29[a]
2 h after detorsion of torsion for 1 h	4.11 ± 0.58	34.75 ± 3.61[a]	92.73 ± 8.75[a]
2 h after detorsion of torsion for 2 h	4.26 ± 0.39[a]	37.74 ± 4.05[a]	82.18 ± 5.18
One sham operation	3.35 ± 0.48	31.88 ± 2.98	79.13 ± 4.06
Two sham operations	3.41 ± 0.51	33.32 ± 3.58	76.30 ± 3.92

[a] $P < 0.05$ compared with basal value group

Table 3. Lactic acid, hypoxanthine and thiobarbituric acid reactive (TBAR) substances values in kidneys (mean ± 1 SD)

Groups	Lactic acid (μmol/g wet tissue)	Hypoxanthine (nmol/g wet tissue)	TBAR (nmol/g wet tissue)
Basal	3.73 ± 0.31	41.51 ± 2.40	76.23 ± 3.90
1 h torsion	3.92 ± 0.65	44.12 ± 5.48	73.35 ± 5.00
2 h torsion	4.31 ± 0.41	46.87 ± 7.26	75.44 ± 5.53
2 h after detorsion of torsion for 1 h	3.75 ± 0.60	49.44 ± 6.15	84.57 ± 7.19
2 h after detorsion of torsion for 2 h	4.18 ± 0.52	48.95 ± 5.63	84.09 ± 9.97
One sham operation	4.05 ± 0.49	44.55 ± 7.95	81.63 ± 9.17
Two sham operations	4.23 ± 0.45	49.89 ± 8.21	82.78 ± 17.09

surgical manipulation of the testis did not cause a change in the intratesticular and intrarenal biochemical parameters studied. Unilateral testicular torsion resulted in an increase in lactic acid, hypoxanthine and TBAR values in the ipsilateral testis. Although the lactic acid, hypoxanthine and TBAR values were significantly lower in the contralateral testes compared with ipsilateral testes, they were significantly elevated compared with basal values. The statistically significant increases in lactic acid, hypoxanthine and TBAR values in contralateral testes after unilateral testicular torsion imply that unilateral testicular torsion causes hypoxia in the contralateral testis also, possibly through a decrease in blood flow as shown by electromagnetic and radioisotopic blood flow measurements [13, 17].

Contralateral testicular blood flow has been shown to decrease after unilateral testicular torsion by electromagnetic flowmeter and ^{133}Xe clearance measurements of blood flow [13, 17]. On the other hand, it has been shown that unilateral varicocele causes an increase in contralateral testicular blood flow [8, 9, 20]. However, the mechanism which causes these changes in contralateral blood flow remains obscure. The stimulus triggering the changes may be transmitted either neuronally or humorally. Studies with experimental varicocele and testicular torsion have shown that the presence of ipsilateral testis is not mandatory for contralateral blood flow changes [2, 8, 9, 20]. Physical distension of the ipsilateral spermatic vein has been speculated to participate in the mechanism behind the contralateral testicular response to varicocele

[7]. Similarly, torted spermatic vessels during testicular torsion might transmit signal to the contralateral blood supply either humorally or neronally [2].

Treatment of testicular torsion by detorsion may further damage the testis. With the resumption of blood flow oxygen is supplied which is necessary for the conversion of hypoxanthine (ATP degradation product) to uric acid in the presence of the enzyme xanthine oxidase [6, 11, 15]. During this conversion abundant free oxygen radicals are produced [6, 11, 15]. These free radicals react with lipids in the cell and mitochondrial membranes forming lipid peroxides [6, 11, 15]. Peroxidation of the lipids in membranes changes the membrane permeability or disrupts membrane integrity and thus cell integrity [6, 11, 15]. This cascade of events is known as "reperfusion injury" [6, 11, 15]. The free radicals cause further cellular damage through the peroxidation of lipids in mitochondrial and cell membranes [6, 11, 15].

Electromagnetic measurement of blood flow has shown that contralateral blood flow increases towards normal after detorsion [17]. A further increase in lipid peroxidation would be expected in both testes after detorsion – which brings about reperfusion. The present study has shown that detorsion after torsion lasting up to 2 h causes an statistically significant further increase in lipid peroxidation in the ipsilateral but not in the contralateral testis. Although detorsion after torsion lasting 1 h caused an increase in lipid peroxide levels in the contralateral testis, this increase was not statistically significant.

The present study investigated intraparenchymal biochemical changes because there are problems in documenting reperfusion injury histopathologically. In order to document reperfusion injury histopathologically, deterioration after detorsion compared with the histopathologic injury after testicular torsion must be demonstrated. Thus testicular histopathology must be studied immediately after testicular torsion; however, a period of at least several days needs to elapse after testicular torsion in order to detect histopathologic changes [3–5, 12]. This period necessary for histopathologic changes to become visible elapses after detorting the testis – which means reperfusing it. Thus the histopathologic changes would then be those of testicular ischemia plus reperfusion instead of those of testicular ischemia alone. Prevention of reperfusion injury can be documented histopathologically by comparing the final injury in a group receiving pharmacologic treatment and an untreated group, but it is impossible to compare histopathologic changes after testicular detorsion with changes after testicular torsion.

Reperfusion injury mediated by oxygen-derived free radicals has been studied extensively in organs other than testis [6, 11, 15]. Bergh et al. [3] tried to prevent reperfusion injury in testis after 60 or 100 min of testicular artery ligation and found that free radical scanvengers did not influence the extent of testicular damage. They postulated that the response of the testis to ischemia and reperfusion may be different from that of other organs. Ischemia induced by artery ligation is a model of global ischemia. Organs vary in their tolerance to ischemia [6, 11]. In organs such as heart and skin, free radical scavengers are effective in preventing reperfusion injury after global ischemia [6]. However, in intestine free radical scavengers fail to prevent reperfusion injury after global ischemia but do prevent it after brief periods of partial ischemia [6]. Testis might resemble intestine in its response to ischemia.

Additionally, reperfusion injury is a combination of ischemic and reperfusion components [15]. In organs intolerant of global ischemia the injury due to the global ischemic component itself may be so great that ablation of the reperfusion component has no significant or clinically relevant effect. Free radical scavengers are effective when the major portion of the total injury sustained is caused by the reperfusion component [15]. Sixty minutes of global ischemia might itself cause a level of damage in testis that leaves no place for further damage in the reperfusion period.

Testicular torsion of 720° initially occludes the veins but not the arteries; this produces partial ischemia in the early periods of torsion. We have studied reperfusion injury in both testes after 6 h of 720° testicular torsion and could find no evidence of reperfusion injury [1]. We therefore postulated that hypoperfusion might persist both in the ipsilateral and contralateral testes after detorsion [1]. This is consistent with other studies. Turner et al. [19] showed that ipsilateral testicular blood flow does not return after 720° testicular torsion lasting more than 4 h; however, it does return after 1 or 2 h of 720° torsion and yet significant histopathologic changes take place in both testes after such short periods of 720° torsion [4, 5, 19]. The present study has similarly shown that ipsilateral blood flow does return after detorsion following 1 or 2 h of 720° torsion; this is characterized by an statistically significant decrease in lactic acid and hypoxanthine and an increase in TBAR in ipsilateral testis.

In clinical practice ipsilateral torted testis may benefit from inhibitors of free radical production or free radical scavengers given before detorsion in the early periods of torsion. Prevention of reperfusion injury is possible using several enzymes such as superoxide dismutase (SOD) and drugs such as allopurinol, ensuring that adequate blood levels are reached before resumption of blood flow [6, 15]. SOD is found tissues and catalyzes the reactions scavenging free oxygen radicals [6, 15]. The pharmacologic activity of allopurinol and its metabolites such as oxypurinol is through inhibition of the enzyme xanthine oxidase [15]. Further testicular damage caused by reperfusion in ipsilateral testis may be attenuated by this approach. Research regarding this issue is in progress in our laboratory.

It is concluded that ipsilateral testicular torsion causes a decrease not only in ipsilateral but also in contralateral testicular perfusion which results in elevated lactic acid, hypoxanthine and lipid peroxide levels. Additionally, detorsion following up to 2 h of 720° torsion causes reperfusion injury in ipsilateral but not in contralateral testes characterized by a further increase in lipid peroxide levels. There appears to be a potential role for free radical scavengers in preventing reperfusion injury in ipsilateral testis after detorsion.

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