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Testicular nitric oxide levels after unilateral testicular torsion/detorsion in rats pretreated with caffeic acid phenethyl ester

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Abstract Nitric oxide (NO) plays an important role in modulating blood flow in normal and in several pathological conditions, and its levels seem to change with ischemia–reperfusion injuries. Caffeic acid phenethyl ester (CAPE), an active component of propolis, exhibits antioxidant properties. This experimental study was designed to determine the changes in NO levels and the effect of CAPE on NO levels after testicular torsion/detorsion in rats. Thirty-five adult male albino rats were divided into four groups: sham operation ($n = 8$), torsion ($n = 9$), saline/detorsion ($n = 9$), and CAPE/detorsion ($n = 9$). Rats in the sham operation group were killed after the testes were handled without torsion. Rats in the torsion group were killed after 720° clockwise testicular torsion for 2 h. CAPE was administered 30 min before detorsion in the CAPE/detorsion group and saline was administered in the saline/detorsion group. After 4 h of testicular detorsion in both of these groups, the rats were killed and bilateral orchiectomy was performed to determine the tissue levels of NO. The level of NO in the torsion group (113.77 ± 33.18 nmol/g protein) was significantly higher than that of the sham operation group (64.53 ± 29.64 nmol/g protein). In the saline/detorsion group, the NO level (31.26 ± 12.58 nmol/g protein) was significantly lower than in the torsion and sham operation groups. CAPE administration in the CAPE/detorsion group seemed to raise the NO level (72.63 ± 23.87 nmol/g protein) above the level of the sham operation group. Contralateral testes were not affected by the torsion/detorsion processes performed on the ipsilateral testes. These results show that NO levels increase with torsion and decrease with detorsion. CAPE administration seems to increase tissue NO levels and this may be important for protecting the testes from torsion/detorsion injuries.

Key words Testicular torsion · Nitric oxide · Caffeic acid phenethyl ester

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Introduction

Testicular torsion has been implicated in testicular injury and infertility. Testicular injury is proportional to the duration and degree of torsion, and detorsion is one of the most important factors in further injury [2]. Testicular injury resulting from torsion and detorsion resembles the phenomenon of ischemia–reperfusion injuries observed in other organs. During the detorsion process, with the resumption of blood flow, a huge amount of molecular oxygen is supplied to the tissues and abundant amounts of free oxygen radicals, which are responsible for reperfusion injury, are produced [4].

Nitric oxide (NO), a water- and lipid-soluble free radical, is generated from L-arginine by the action of nitric oxide synthases (NOSs) and plays an important role in modulating blood flow and tissue injury in normal and in several pathological conditions. Ischemia causes an increase in NOS activity in the vascular endothelium and, later, in a range of cells including infiltrating neutrophils and macrophages. While NO levels increase during ischemia, reperfusion leads to the generation of the superoxide radical (O_2^-) through the action of several mechanisms [18]; the interaction between NO and O_2^- produces peroxynitrite ($ONOO^-$). The breakdown of NO by O_2^- anions may be a factor in the decreased NO levels [5]. Despite intensive research, there remains uncertainty about the role of NO in post-ischemic tissue injury and also as to when, and indeed whether, NO production is increased or decreased.

Propolis, a natural hive product collected by honeybees, has strong antimicrobial, anti-inflammatory, antioxidant, and antineoplastic activities. Caffeic acid phenethyl ester (CAPE), an active component of propolis, may be responsible for most of these biological activities [13–16] (Fig. 1). At a concentration of 10 μM, CAPE completely blocks the production of reactive oxygen species in human neutrophils and in the xanthine/xanthine oxidase (X/XO) system [29].

The aim of the present study was to investigate the changes in the levels of NO and the effects of CAPE on NO levels during temporary testicular torsion/detorsion in rats.

Materials and methods

Thirty-five adult male albino rats weighing 210–240 g were divided into four groups. All surgical procedures were performed while the rats were under intramuscular ketamine (50 mg/kg) anesthesia. Torsion, detorsion, and sham operations were performed through standard ilioinguinal incisions. The rats, except for those in the sham operation group, were subjected to left unilateral testicular torsion (720° rotation in the clockwise direction), excluding the epididymis, for 2 h. The twisted testes were fixed to the scrotum by a silk suture through the tunica albuginea in the torsion and detorsion groups. In the sham operation group, the testes were brought through the incisions and replaced and a silk suture was placed through the tunica albuginea.

Sham operation group ($n = 8$). After preparation of the testes, the rats were killed and bilateral orchiectomy was performed.

Torsion group ($n = 9$). The aforementioned testes were removed after 2 h of torsion.

Saline/detorsion group ($n = 9$). Saline was injected intraperitoneally (i.p.) 30 min before detorsion. The rats were killed after 4 h of reperfusion and the testes were removed.

CAPE/detorsion group ($n = 9$). CAPE (10 μmol/kg), which was previously demonstrated to be absorbed trans-serosally [16], was injected i.p. 30 min before detorsion. After 4 h of detorsion, the rats were killed and bilateral orchiectomy was performed. The CAPE applied in this study was synthesized according to the technique described by Grunberger et al. [11].

All organs were washed twice with cold saline solution, placed into glass bottles, labeled, and stored in a deep freeze (−30 °C) until processing (about 3 days). After the testes were cut into small pieces, tissues were homogenized in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.50 ml/l Triton X-100 with a homogenizer (Tempest Virtishear, Model 278069; The Virtis Company, Gardiner, NY, USA) for 2 min at 5,000 rpm. All procedures were performed at +4 °C. NO levels were determined in the homogenate. Protein concentrations in the homogenate were determined according to Lowry et al.'s method [19].

Since tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels can be used to estimate NO production, we measured the concentrations of these stable NO oxidative metabolites. Quantitation of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO_2^- with a mixture of naphthlethylenediamine and sulfanilamide [6]. Samples were deproteinized with Somogyi reagent [28]. Following cleanup, an aliquot of the sample was mixed with fresh reagent, and the absorbance was measured in a spectrophotometer (Ultraspec Plus, Pharmacia LKB Biochrom, UK) to give the NO_2^- concentration. For NO_3^- detection, a second aliquot was treated with copperized cadmium (Cd) in glycine buffer at pH 9.7 (2.5–3 g of Cd granules for a 4 ml reaction mixture) to reduce NO_3^- to NO_2^-. The concentration of NO_2^- in this aliquot thus represented the total NO_3^- plus NO_2^-. The NO_3^- concentration in the sample was thus given by the difference between the two aliquots. A standard curve was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/l) of sodium nitrite. Linear regression was done using the peak areas from the NO_3^- and NO_2^- standards. The resulting equation was then used to calculate the unknown sample concentrations. All chemicals used in this assay were obtained from Sigma, with the exception of the Cd granules (Fluka). Results are expressed as nmol/g protein.

Data are expressed as means ± standard deviation (SD). All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, Chicago, IL, USA). Nonparametric analyses with the Mann–Whitney *U*-test were performed on the biochemical variables data. Correlation analyses in each group were tested with Spearman's test. *P* values of less than 0.05 were considered to be significant.

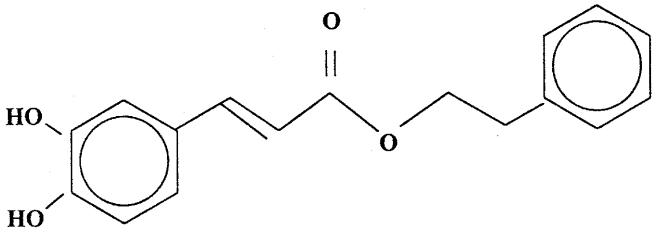

Fig. 1 Chemical structure of CAPE

Results

NO levels for ipsilateral twisted (left) and contralateral nontwisted (right) testes are shown in Fig. 2. The NO level in the sham operation group was 64.53 ± 29.64 nmol/g protein. This level was the mean value of both unprocessed testes (left and right). In the torsion group, the NO level (113.77 ± 33.18 nmol/g protein) was significantly higher than that of the sham operation group ($P < 0.005$), whereas there was a significant decrease in the saline/detorsion group (31.26 ± 12.58 nmol/g protein; $P < 0.01$). In the CAPE/detorsion group, the NO level

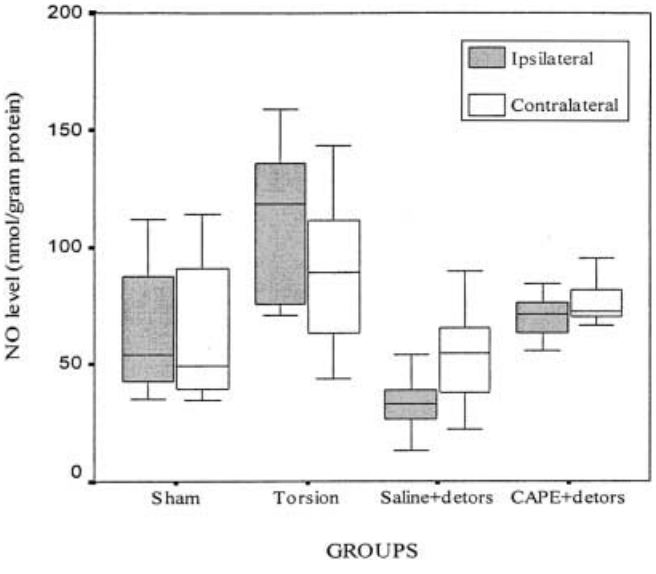

Fig. 2 Nitric oxide (NO) levels in the testicular tissue of different groups of rats. NO levels are higher in ischemia and lower in ischemia-reperfusion groups than in sham operation and CAPE groups ($P < 0.005$)

(72.63 ± 23.87 nmol/g protein) increased and returned to a value that was higher than the original level.

There was no significant difference in NO levels between the contralateral testes. Correlation analyses did not show any positive intracorrelations between NO levels of the ipsilateral and contralateral testes in three experimental groups, the exception being the sham operation group.

Discussion

Results of the present study indicate that testicular torsion and detorsion induce significant changes in NO levels as a result of both ischemia and reperfusion. We did not find any detailed study in which NO levels have been studied in both ipsilateral and contralateral testes after the testicular torsion and detorsion process. In our study, while unilateral testicular torsion resulted in significant changes in NO levels in the ipsilateral testis, NO levels in the contralateral testes were not affected in any of the groups. Many laboratories previously showed that unilateral testicular torsion had an adverse effect on the contralateral testis [7, 17, 26]. Our results did not reveal any adverse effect on the contralateral testis in terms of NO levels.

In the present study, we have demonstrated that ischemia increases the concentrations of testicular NO_2^- and NO_3^-, which are indicative of the endogenous overproduction of NO. High tissue levels of NO in the ischemia group compared to the sham operation group (approximately twofold) suggest that NOS activity may increase under such abnormal conditions, i.e. hypo-oxygenation [24]. Other possible explanations for the elevation of tissue NO concentrations are the sequestration of activated neutrophils to the testis [1] or the formation of NO by an NOS-independent mechanism. In another study [21], XO was reported to catalyze the reduction of NO_3^- and NO_2^- to NO under hypoxic conditions [21]. There are several reports suggesting a protective role for NO in ischemic tissues such that the inhibition of NO and NOS was demonstrated to exacerbate the oxidative damage [31, 32]. Furthermore, while the infusion of L-arginine was beneficial in renal ischemia [30], exogenous NO administration improved renal function and diminished inflammatory responses in a kidney subjected to an ischemia-reperfusion process [8]. Our results support the view that increased generation of NO may be important in the redistribution of blood flow to ischemic tissues, and this may be the result of the vasodilator role of NO in ischemia [33].

Our results also reveal that NO levels decrease with the detorsion process. It is well documented that during reperfusion, with the resumption of blood flow, huge amounts of molecular oxygen are supplied and abundant amounts of free oxygen radicals are produced. A decrease in NO levels after reperfusion may suggest that NO is consumed by the free oxygen radicals. There are several reports supporting this view. When cultured pulmonary epithelial cells were subjected to acute oxidative stress, NO was shown to be inhibited by oxygen radicals [12]. In another study [3], reactive oxygen species inactivated the relaxing NO and promoted a reduction in blood flow. Attenuation in NO-mediated vasodilatation was reported to be dependent on the inactivation of NO by O_2^- anions generated by endothelial XO [27]. Since NO interacts with free oxygen radicals, the marked decrease in NO levels after reperfusion in our study may be the result of oxidant species which react with NO, possibly causing a decrease in NO concentration in the reperfused tissue. This suggests that NO may be a protecting factor against free oxygen radical toxicity. We may conclude that oxygen radicals downregulate NO synthesis, contributing to vasoconstriction in reperfusion injury [23].

Tissue or body fluid concentrations of NO_2^- and NO_3^- have been used in several clinical studies as a means of measuring endogenous NO production [22]. While NO probes have been developed [20], their use has been restricted to in vitro/ex vivo studies. Since the indirect measurement of NO via the metabolites NO_3^- and NO_2^- is an established technique [9, 10], we chose to measure NO_2^- and NO_3^- in order to determine NO levels in the present in vivo study.

Our results showed that CAPE has a well-distributed systemic circulation and that it has an in vivo effect in addition to its proven in vitro effect [25]. We also demonstrated that CAPE administration brought the NO levels above the sham values, which may be important in protecting tissue from injury. Although tissue NO levels were clearly increased by CAPE, the mechanism is not clear. There are several possibilities: (1) CAPE may directly increase NOS activity; (2) CAPE may activate the production of enzymatic cofactors; or (3) CAPE may selectively activate neutrophil sequestration to the testes, inducing subsequent NO production by the activated cells. Other explanations for the effect of CAPE might be the scavenging of the free oxygen radicals by the other antioxidant enzymes and preventing the inhibition of NO. Since the antioxidant properties of CAPE are well documented, the latter suggestion seems to be more likely. These possibilities have so far not been tested in experimental studies.

In conclusion, testicular torsion results in increased levels of NO in the ipsilateral testis, but not in the contralateral testis. The administration of CAPE before testicular ischemia prevents the decrease in NO levels during reperfusion; thus, CAPE may be useful in dealing with ischemia-reperfusion injuries.

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