Preliminary report on nitric oxide-mediated oxidative damage in adolescent varicocele

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BACKGROUND: The current study was designed to characterize the process of nitric oxide (NO) and peroxynitrite generation through the determination of nitrotyrosine concentration in the dilated veins of varicoceles in adolescents. METHODS: Ten adolescents with a median age of 13 years (range 12–17) affected by a left idiopathic varicocele (grade II and III) were studied. Whole blood samples were withdrawn from a peripheral vein at time of induction of anaesthesia, and from a dilated spermatic vein before ligation. Peripheral blood samples from five adolescents undergoing minor surgical procedures were used as controls. The nitrotyrosine concentration was evaluated by a sandwich enzyme-linked immunosorbent assay (ELISA), using a monoclonal anti-nitrotyrosine antibody and Western blot analysis. RESULTS: Plasma nitrotyrosine concentrations were significantly greater in the spermatic vein when compared with the peripheral vein (P = 0.031). Nitrotyrosine in plasma of controls did not show any significant difference in comparison with peripheral samples from varicocele patients. Western blot analysis confirmed the above data. CONCLUSIONS: In adolescents with a varicocele, there is an increase in nitrotyrosine concentration within the spermatic vein that can cause protein nitration and cytotoxicity via its reaction with various molecular targets. This could have repercussions on both sperm and testis function. We conclude that an oxidative stress status is present and should be considered as an indication for varicocele treatment in the adolescent.

Key words: adolescent/nitric oxide/nitrotyrosine/reactive nitrogen species/varicocele

Introduction

Nitric oxide (NO) has been reported to be increased in the spermatic veins of men affected by varicocele (Mitropoulos et al., 1996; Ozbek et al., 2000). Moreover, in sub-fertile men with varicocele an increase in active metabolites of NO such as peroxynitrite (ONOO⁻) and S-nitrosothiols has been also recorded, suggesting a possible role for NO in sperm dysfunction (Mitropoulos et al., 1996).

Similar results have been also demonstrated in adolescents with grade II and III varicocele, and up-regulated function of the inducible isoform of the enzyme nitric oxide synthase (iNOS) has been identified in the Leydig cells of these patients (Romeo et al., 2001; Santoro et al., 2001).

Other reports in adults with varicocele have recently demonstrated a role for reactive oxygen species (ROS), i.e. hydrogen peroxide, hydroxyl radical and superoxide anion, in causing sperm dysfunction (Hendin et al., 1999; Koksal et al., 2000). Varicocele reduces the antioxidant defences of both seminal and blood plasma causing an oxidative stress status (Barbieri et al., 1999).

An equivalent demonstration of NO-dependent damage due to reactive nitrogen species (RNS) in varicocele patients is still lacking.

Recently, nitrotyrosine has been identified as a marker of NO damage in vivo in patients with different inflammatory diseases (Ter Steege et al., 1998; Sittipunt et al., 2001). Protein tyrosine nitration represents a marker for accurate definition of production and action of peroxynitrite, the active metabolite of NO, in biological elements and also contributes to the toxicity mediated by NO and peroxynitrite.

In order to investigate whether NO tissue injury in adolescent varicocele may at least partially be due to NO-derived reactive species, the current study was designed to characterize the process of NO and peroxynitrite generation through the determination of nitrite/nitrate (NOx) and nitrotyrosine concentration in the dilated veins of adolescents with a varicocele. Demonstration of an increased concentration of nitrotyrosine in the blood from the spermatic vein of varicocele patients could represent a marker of the potential harmful effects of NO.
Materials and methods

Ten adolescent patients with left idiopathic varicocele of grade II or III, after Horner (Horner, 1960), were enrolled in the study. Their ages ranged between 12 and 17 years (median 13). Patients were treated using a microsurgical inguinal varicocelectomy technique. Having gained informed consent, whole blood samples were withdrawn from a peripheral vein at the time of induction of anaesthesia, and from a dilated spermatic vein before any manipulation of the cord and ligation. Peripheral blood samples from five adolescents undergoing minor surgical procedures were used as controls.

Plasma NOx concentrations

Nitrite/nitrate concentrations were measured in the plasma as reported previously (Misko et al., 1993). After conversion of nitrate to nitrite by nitrate reductase, sample extracts were reacted with 2,3-diaminophenanthrene (0.032 mmol/l in 0.62 mol/l HCl). Fluorescence was determined using a model LS-3B Perkin Elmer fluorescence spectrometer with excitation at 365 nm and emission at 450 nm.

Nitrotyrosine ELISA

Nitrated plasma proteins were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) (Ter Steege et al., 1998). In brief, 96-well plates (Nunc-Immunoplate; MaxiSorp, Roskilde, Denmark) were coated with 1 μg/ml monoclonal antibody to nitrotyrosine (HM11; Hbt, Uden, Netherlands) in PBS (100 μl/well) overnight at 4°C. After blocking with 1% bovine serum albumin (BSA) (150 μl/well) and washing, nitrated proteins were added as antigen (100 μl/well) for 1 h. The plates were then incubated with biotinylated HM11 (Hbt) diluted 1:1000 in PBS containing 0.1% BSA (100 μl/well) for 1 h. Thereafter, 0.1 μl of streptavidin–horseradish peroxidase conjugate (1:1000; Amersham Biosciences, Buckinghamshire, UK) was added to each well, and the plate was incubated for 3 h at 37°C. The plates were washed three times with washing buffer to completely remove any reagents not bound to the solid phase. Finally, 0.1 μl of O-phenylenediamine substrate solution was added to each well to develop a yellowish colour. The enzymatic reaction was stopped by addition of 0.1 μl/well of 2 Normal H2SO4. The amount of each well to develop a yellowish colour. The enzymatic reaction was completed remove any reagents not bound to the solid phase. The plates were washed three times with washing buffer to completely remove any reagents not bound to the solid phase. Finally, 0.1 μl of O-phenylenediamine substrate solution was added to each well to develop a yellowish colour. The enzymatic reaction was stopped by addition of 0.1 μl/well of 2 Normal H2SO4. The amount of each well to develop a yellowish colour.

SDS–PAGE and Western blotting

Gel electrophoresis of plasma proteins was performed on an 8.5% polyacrylamide gel using a previously described buffer system (Laemmli, 1970). Two identical gels were run at the same time. One was submitted to Coomassie blue staining and the other was used for blotting onto a transfer membrane.

When the transfer was complete, the nitrocellulose membrane was blocked for 1 h with 5% non-fat milk dissolved in 20 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl and 0.5% Tween 20. Next the membrane was incubated with monoclonal antibody to nitrotyrosine (1:500, HM11; Hycult Biotechnology, Uden, Netherlands) for 1 h shaking at 37°C. After washing, the blots were incubated with peroxidase-conjugated goat anti-murine antibodies (1:1000; Amersham Biosciences). The nitrated proteins were visualized using chemiluminescence reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and a 20–30 s exposure to autoradiography film.

Statistical analysis

Data are expressed as medians and 95% confidence intervals (CI). A non-parametric paired Wilcoxon test was used for comparison between groups. Spearman’s rank correlation was used to determine whether nitrotyrosine concentrations significantly correlated with the age of patients or with time of onset of symptoms. Probability levels <0.05 were considered significant.

Results

The median concentrations of NOx in the serum from the spermatic veins showed a 4-fold increase when compared with those in peripheral veins; 9.26 nmol/ml (95% CI 5.3–13.8) versus 2.32 nmol/ml (95% CI 1.5–3.3) respectively (P = 0.031; Figure 1).

Median plasma nitrotyrosine concentrations were significantly greater in the spermatic vein when compared with the peripheral vein; 12.5 nmol/l (95% CI 1.2–27.9) versus 0.87 nmol/l (95% CI 0.17–1.23) (P = 0.031; Figure 2). Nitrotyrosine in plasma of controls 0.73 nmol/l (95% CI 0.15–1.8) did not show any significant difference in comparison with peripheral samples of varicocele patients. The increase in nitrotyrosine was not correlated with the age of patients at surgery (r = −0.66; P = 0.23) or with the reported time of onset of symptoms (r = 0.47; P = 0.45).

Higher concentrations of nitration of plasma proteins in the spermatic vein in comparison with peripheral blood of patients with left idiopathic varicocele were confirmed by SDS–PAGE and Western blotting. Nitrotyrosine was almost undetectable in
the plasma from peripheral blood (lane p in D, E and F of Figure 3). In contrast, positive staining of the blots was detected in the proteins with a relatively high molecular weight (>66 kDa) from spermatic blood (lane s in D, E and F of Figure 3).

Discussion

In the reproductive system, different roles have been ascribed to NO in both male and female biology (Rosselli et al., 1998). In particular, in males, different isoforms of NOS have been identified in Sertoli cells, Leydig cells, peritubular lamina propria, endothelial cells of testicular vessels, epididymis and vas deferens, suggesting a definite role in contractile, haemodynamic, hormonal aspects of testicular and epididymal function as well as in spermatogenesis and germ cell degeneration (Zini et al., 1996; Middendorf et al., 1997; Fujijsawa et al., 2001; Santoro et al., 2001). The effects mediated by NO are dose-dependent; at physiological concentrations it acts as the mediator of the aforementioned functions, while at supra-physiological concentrations it can become harmful to the reproductive system (Rosselli et al., 1995; Del Punta et al., 1996; Kostic et al., 1998).

The harmful effects of NO are mediated by biologically activated molecules produced by the reaction of NO with the superoxide anion yielding ONOO⁻ and peroxynitratic acid (ONOOH). These are strong oxidant molecules that can cause molecular damage to a variety of tissues. The acid ONOOH reacts with the cysteine residues of proteins or glutathione, forming S-nitrosothiols. Peroxynitrite also modifies the tyrosine residues, producing nitrotyrosine (Radi et al., 2000). This last has been reported to be increased in different inflammatory conditions (Ter Steege et al., 1998; Sittipunt et al., 2001) as well as in conditions of hypoxia–ischaemia (Tan et al., 2001).

There are different methods to measure nitrotyrosine. In this study, a semi-quantitative assay for nitrated plasma proteins recently developed using a monoclonal antibody and ELISA technique was used. This technique has been reported to quantitate the NO mediated systemic damage, as demonstrated in patients with coeliac disease (Ter Steege et al., 1998).

In the present study, the concentration of NOx, i.e. the end products of NO metabolism, was first determined. A significant increase of NOx in the blood of spermatic veins from varicocele patients was noted, as previously demonstrated (Figure 1). A possible source for the overproduction of NO has been recently identified in Leydig cells of adolescents with varicocele. Immunohistochemical and Western blot analysis have demonstrated an up-regulation of the inducible isoform of the enzyme iNOS in Leydig cells (Santoro et al., 2001). The NO produced at supra-physiological concentrations can freely diffuse across membranes and exert action through its biologically activated molecules at different levels.

An attempt was also made to verify whether or not the increased concentration of NO could produce more reactive species with potential negative effects on the reproductive system. The concentration of nitrotyrosine, a biological marker of NO-induced damage, was investigated. Indeed, the concentration of nitrotyrosine in the spermatic veins was significantly higher compared with the peripheral veins of the same patients (Figure 2), indicating that NO causes nitration of plasma proteins with higher molecular weight. This condition was not correlated with the age of patients at surgery (r = –0.66; P = 0.23) or with the reported time of onset of symptoms (r = 0.47; P = 0.45).

The selective modification of tyrosine residues may have profound effects on the biological functions of the testis. S-nitrosoylation causes deregulation of cellular signal transduction processes; it also has harmful effects on cellular energetics through the inhibition of complex I in mitochondrial respiration (Szabò, 2000). Moreover, in blood vessels nitrotyrosine has been recognized to cause selective vascular endothelial dysfunction through the promotion of DNA damage and/or apoptosis (Mihm et al., 2000), which could exacerbate vasodilatation in this anatomical area (Jin et al., 2001).

Different mechanisms, both enzymatic and proteolytic, normally protect cells against protein nitration. In persisting pathogenic conditions that generate increased amounts of RNS, such as occurs in varicocele, the repair mechanisms may be overwhelmed, resulting in a pathological outcome (Szabò, 2000). Recently an increased concentration of carbonyl groups has been demonstrated in the spermatic blood of young adult
males with clinical and sub-clinical varicocele, suggesting oxidative damage also to plasma proteins (Chen et al., 2001).

The effect of varicocele on male fertility is still a matter of debate (Jarow, 2001). The clinical evidence available suggests that the effect seems to vary according to age. In adolescents, different clinical studies support the hypothesis that the effect of varicocele is progressive. In particular, hypotrophy has been demonstrated in the left testis of intermediate grade varicocele, and bilateral hypotrophy for high grade ones (Kass et al., 2001). Catch-up growth has been also documented by prospective studies after varicocele repair (Podeszta et al., 1994; Paduch and Niedzielski, 1997). Surgery in adolescents may also result in maintenance of fertility potential (Pozza et al., 1994). Other studies have demonstrated ultrastructural modifications of the lamina propria and basal lamina, similar to but less severe than that observed in adults (Santoro et al., 1999, 2000). In contrast, the clinical data available do not support a similar progressive effect of varicocele in adulthood (Jarow, 2001).

The results of this study describe a condition of NO-mediated oxidative stress limited to the testis of adolescents with varicocele. Systemic stress, as described in adults, was not observed.

Our data support the need for surgical treatment in adolescent varicocele to interrupt the destructive cycle that causes blood stasis, NO overproduction and nitrotyrosine formation. Treatment options should also consider antioxidant therapies to counteract the oxidizing effects of both ROS and RNS (Lenzi et al., 1996, 2000).

References

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