L-arginine augments oxidative stress in cryptorchid testes of adult Sprague-Dawley rats

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Accepted 25 March, 2011

Experimental cryptorchidism has been associated with increased apoptotic degeneration of spermatogenic cells resulting in increased free radical production. The aim of this study is to determine the effect of L-arginine on semen parameters, histology and the antioxidant activities on induced cryptorchid rat testis. Twenty male Sprague-Dawley rats weighing between 150-250 g were divided into four groups (A- D) each with 5 rats per group. Animals in groups A and C were made unilaterally cryptorchid by anchoring the upper pole of the testis to the anterior abdominal wall. While group A was treated with 15 mg/100 mg body weight of L-arginine, group C had no treatment. Group B animals received the same dose of L-arginine but not made cryptorchid. Group D animals served as control and received distilled water. A significant decrease (p<0.05) in the sperm count was observed in groups A, B and C when compared with the control. Sperm motility declined significantly in groups A and C. Seminiferous tubular atrophy, depletion of spermatogenic cells as well as infiltration of interstitial spaces was common in cryptorchid rats as well as those treated with l-arginine. Serum testosterone levels were significantly increased in group B whereas group C recorded a decline. Luteinizing hormone levels were significantly increased in groups B and C whereas follicle stimulating hormone levels were decreased in group C when compared with the control. MDA levels were significantly increased in groups A and C while SOD activity declined significantly in these groups. Catalase activity decreased significantly in group C when compared with the control while glutathione reductase activity reduced significantly in groups A and C when compared with the control. These results indicate that administration of 15 mg/100 mg BW of L-arginine causes testicular oxidative stress and do not improve the outcome of cryptorchidism in adult male Sprague-Dawley rats.

Keywords: Cryptorchidism, l-arginine, testis, histology, antioxidants, hormones.

INTRODUCTION

Cryptorchidism results from failure of the testis to descend into the scrotum. It is a primary congenital illness affecting up to 3% of boys (De Miguel et al., 2001) and a major risk factor for male infertility. In most male mammals the scrotal temperature is always lower than that of the abdomen hence allowing for the maintenance of optimal environment for testicular function. Cryptorchidism has been shown to induce heat stress (Yin et al., 1997) which predispose to free radical formation that is responsible for the infertility in these patients. The surgical induction of cryptorchidism in experimental animals has been shown to cause disruption of spermatogenesis leading to infertility (Saalu et al., 2007).

L-arginine is the biological precursor of nitric oxide (NO), an endogenous gaseous messenger molecule involved in a variety of endothelium-dependent physiological functions (Wu and Meininger, 2000) including its critical role in cardiovascular protection and immune support (Sunita et al., 2000). It is now evident that an increase in NO and a decrease in total antioxidant capacity occur with cryptorchidism and contribute to accelerated germ cell loss. Administration of nitric oxide synthase inhibitor, N-omega-nitro-L-arginine methyl ester, attenuated apoptosis and improved spermatogenesis in
L-arginine decreases oxidative stress by reduction of the vascular superoxide anion production with improvement of endothelial function in hypercholesterolaemic subjects (Kawano et al., 2002). In several conditions where oxygen metabolites are thought to mediate endothelial and myocardial injury, L-arginine has protective effects that might be due to its direct chemical interaction with oxygen radicals (Lass et al., 2002). Moreover, dietary L-arginine supplementation attenuates the oxidative stress induced by burn injury with a better macrophage response (Tsai et al., 2002). These beneficial effects of L-arginine have been attributed to its dependent formation of NO within the endothelial lining (Brandes et al., 2000).

Men with unilateral undescended testis have been shown to father significantly more children than those with bilateral undescended testes (Cendron et al., 1989) and studies have pointed to the fact that unilateral cryptorchidism induces germ cell apoptosis in the ipsilateral testis (Wang et al., 1998; Zini et al., 1999; Watts et al., 2000); attention is now focused on the possible intervention of L-arginine in the treatment of persistent cryptorchidism since it is required for normal spermatogenesis (Tanimura, 1967). The outcome would provide a better prognosis in terms of testicular function in adulthood (Hadziselimovic, 2001). This work is therefore conceived to determine the effects of L-arginine supplementation on a unilateral cryptorchid testis of matured male Sprague-Dawley rats.

MATERIALS AND METHODS

Chemicals

L-arginine and ketamine were purchased from Sigma Chemical (St Louis, MO). 15 mg/100 mg body weight L-arginine was orally administered to the rats via a metal oro-pharyngeal canula.

Animals

Twenty male Sprague-Dawley rats weighing 150-250 g were used for the study. The animals were procured from the Animal House of the College of Medicine, University of Lagos and acclimatized for three weeks before commencement of experiment. Animals were housed in the departmental animal house at temperature of 28±2°C with 12 hour light/12 hour dark cycle. Commercial rat feed was sourced from the Livestock feeds Ltd, Ikeja and water provided ad libitum. Animals were randomly divided into four groups (A – D) consisting of 5 rats per group.

Experimental protocol

Groups A to C were the experimental groups; in group A the right testis was made cryptorchid and 15 mg/100 g L-arginine administered for 8 weeks. Group B was treated with 15 mg/100 g L-arginine for 8 weeks while in Group C right testis made cryptorchid only with no treatment. Group D served as the control and the rats were neither rendered cryptorchid nor treated with L-arginine.

To induce right cryptorchidism, each rat was anaesthetized and a small incision was made in the upper abdomen. General anesthesia was given with 10 mg/kg of ketamine (Tsai et al., 2002). The right testis was gently brought up into the abdominal cavity and fixed on the upper abdominal wall using a 4-0 Nylon suture passing through the connective tissue of the caput epididymis as previously described in Jegou et al. (1984). All the animals subsequently recovered fully. At the end of the experimental period, all rats were sacrificed the next day by cervical dislocation and the testes harvested following a laparotomy.

Estimation of plasma levels of Testosterone, Luteinizing hormone and follicle stimulating hormone:

Blood was collected by ventricular puncture into heparinized bottles and centrifuged at 3000 rpm for 10 minutes using a bench centrifuge at 25°C to obtain the plasma. Testosterone, LH and FSH was assayed using the ELISA method using kits from Biotec Laboratories Ltd, UK. Plasma samples collected were stored at -20°C.

Epididymal sperm count, motility and morphology

The testes from each rat were carefully exposed and removed, trimmed free of the epididymis and adjoining fatty tissues. Caudal part of the epididymis was removed and placed in a beaker containing 1mL physiological saline solution whereupon it was minced using a pair of sharp scissors. It was kept for a few minutes to allow the spermatozoa swim out into the solution. Sperm count and motility were determined as described in Saalu et al. (2008). Semen drops were placed on the slide and two drops of warm 2.9% sodium citrate were added. The slide was covered with a cover slip and examined under the microscope using X40 objective for sperm motility. Sperm count was done under the microscope using the improved Neubauer’s counting chamber (Haemocytometer). The sperm concentration was then calculated and recorded in million and expresses as (X) x 10^5/ml, where X is the number of sperm in a 16-celled square. Sperm morphology was evaluated using the light microscope at X400 magnification. Caudal sperm were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada). Five hundred sperm from the
Figure 1: Histological sections of testis of rats H & E X 40. A-rt shows right cryptorchid testis seminiferous tubule devoid of spermatogenic cells (arrowed), interstitial spaces filled with inflammatory cells. A-lt shows contralateral left testis of same group with normal spermatogenic cells in seminiferous tubules. B shows normal histo-architectural patterns of testis of group B rats. C-rt shows right cryptorchid testis with few basal spermatogonia (arrowed).

sample were scored for morphological abnormalities (Atessahin et al., 2006). Briefly, in wet preparations using phase-contrast optics, spermatozoa were categorized. In this study, a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head and was expressed as a percentage of morphologically normal sperm.

**Enzyme assay**

Testicular tissue was homogenized in Teflon-glass homogenizer with appropriate volume of phosphate buffer 0.1M, pH 7. The samples were then centrifuged at 3500 rpm for 20 minutes in a refrigerated centrifuge. The supernatant was collected and used for determination of activities of superoxide dismutase (SOD), catalase, glutathione reductase and malondialdehyde (MDA). SOD was determined as described in Sun and Zigma (1978), catalase activity determined according to the method of Aebi (1983). Reduced glutathione was estimated according to the method of Sedlak and Lindsay (1968). MDA was determined using the method of Buege and Aust (1978).

**Statistical analysis**

Data was analyzed using the Student’s T-test and expressed as mean ± SD. P value of p<0.05 was considered significant.

**RESULTS**

**Histological results**

Group A: There was necrosis of the spermatogenic cells in the right cryptorchid testis and inflammation in the interstitium. The contralateral left testis was normal in histoarchitecture (Figure 1).

Group B: There was normal histological architecture of the seminiferous epithelium and interstitial spaces were essentially normal.

Group C: The right cryptorchid testis showed depletion of spermatogenic cells in the seminiferous tubules with mild inflammation in the interstitial spaces. The left contralateral testis showed normal histological appearance (Figure 2).

Group D: Testicular sections of control rats showed 3-5 cell thickness of spermatogenic cells in the seminiferous
Figure 2: Histological sections of testis of rats H & E x 400. C-It shows contralateral left testis of group C with normal seminiferous epithelial lining similar to control group D.

Table 1: Seminal fluid analysis of treatment groups

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>Treatment</th>
<th>Sperm count (x10⁶/ml)</th>
<th>Sperm motility (%)</th>
<th>Sperm morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cryptorchid + L-arginine</td>
<td>15.00 ± 8.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.60 ± 26.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.04 ± 9.24</td>
</tr>
<tr>
<td>B</td>
<td>L-arginine</td>
<td>15.20 ± 9.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.60 ± 25.00</td>
<td>14.30 ± 3.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>Cryptorchid only</td>
<td>11.50 ± 4.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.00 ± 11.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.50 ± 3.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>DW</td>
<td>26.30 ± 2.59</td>
<td>87.60 ± 8.62</td>
<td>7.00 ± 2.65</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SD; <sup>a</sup>: P< 0.05; n=5; DW is distilled water

Table 2: Results showing hormonal levels in treated groups

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>TT (nmol/L)</th>
<th>LH (IU/L)</th>
<th>FSH (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.52 ± 2.10</td>
<td>0.18 ± 0.11</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>B</td>
<td>10.72 ± 2.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>C</td>
<td>4.47 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>5.90 ± 0.22</td>
<td>0.30 ± 0.06</td>
<td>0.53 ± 0.26</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SD; <sup>a</sup>: P< 0.05; n=5; TT is testosterone; LH is luteinizing hormone; FSH is follicle stimulating hormone

tubules with interstitial spaces that were essentially normal.

Significant decline in sperm count and motility (P<0.05) was observed in all treated groups when compared with control but this was maximal in group C. Similarly, sperm morphological abnormalities (round head, double head, no tail) was significantly higher (Table 1) in groups B and C compared with control.

TT was significantly increased in group B and declined in group C while group A value was high but not significant when compared with control. Similar trend was observed in LH values as well. FSH levels were maximally depressed in group C whereas the other groups were not significantly affected (Table 2).

MDA: A significant increase in MDA levels was recorded in groups A and C when compared with the control (Table 3).

SOD: A significant decline was recorded in the activity of SOD in groups A and C compared with control. Though the value for group B was high, it was not significant (Table 3).

CAT: Catalase activity declined significantly in group C while group A was not significant (Table 3).

GSH: Groups A and C recorded a significant decline in GSH when compared with control (Table 3).

DISCUSSION

The significant decline in sperm count in cryptorchid rats as well as those that received L-arginine supplementation is an indication that unilateral cryptorchidism with L-arginine supplementation severely impaired spermatogenesis (Ahotupa and Huhtaniemi, 1992).
Necrosis of spermatogenic cells in the seminiferous tubules, seminiferous tubular atrophy as well as interstitial inflammation was observed in the histological sections of cryptorchid testis after L-arginine treatment and in testes of cryptorchid rats. Elevated abdominal temperatures as a result of the experimentally induced cryptorchidism may have been responsible for this observation as retention of the testes inside the abdomen results in disruption of spermatogenesis (Zhang et al., 2002). L-arginine treatment exacerbated the histological disruptions following experimental cryptorchidism in this study. Previous study by Ozokutan et al. (2000) had showed that in mice pre-treated with N-mono methyl L-arginine, which acts by inhibiting NO synthesis, adverse histological effects were absent.

L-arginine is a precursor of nitric oxide, a free radical (Bechman and Koppenol, 1996) which is reported to cause decreases in antioxidant capacity and germ cell apoptosis seen in cryptorchid testes (Chandral., 2009). This is observed by the decline in sperm count in groups A and B that had L-arginine supplementation and L-arginine alone respectively. NO could possibly have induced the cytotoxic effects on the testes of these rats. Again, spermatozoa motility is a high energy-dependent process and NO have been shown to inhibit the ATP levels by inhibiting the enzymes involved in electron transport (Dimmeler et al., 1992). Hence, the low sperm motility observed in L-arginine treated rats could be due to oxidative stress-induced damage via NO production following L-arginine treatment. Cryptorchid rats equally had low sperm motility which is in agreement with previous studies by Grasso et al. (1991). Abnormal sperm rates seen in L-arginine treatment further alludes to oxidative stress-induced damage while cryptorchidism in rats have been observed to induce similar aberrations as reported by Jones et al. (1977). That the epididymis of the cryptorchid testes had significantly lower sperm parameters than the unmanipulated testes confirms the deleterious effect of cryptorchidism on testicular functions.

Testosterone synthesis is primarily from the Leydig cells in the interstitium. LH is the major trophic stimulus for the synthesis of TT. L-arginine treated rats showed high levels of LH and TT whereas in cryptorchid rats TT was significantly depressed. The reason for this could be attributed to heat stress induced by cryptorchidism (Yin et al., 1997). Kostic et al., 1998 previously reported that a short immobilization stress in rats produced a sharp decline in serum testosterone levels and blocked the LH-induced TT production. Also it is possible that the failure of the negative feedback mechanism resulting from Leydig cell failure to produce optimal TT could be responsible for this observation as supported by Saalu et al., 2007). This suggests that although all cell types of the testes were affected by cryptorchidism, the most prominent damage occurred in the seminiferous epithelium. Thus the Leydig cells, which secrete testosterone, were minimally affected by experimental cryptorchidism. The significant decline in FSH levels in cryptorchid rats could be attributed to the hypo-function of Sertoli cells in these rats (Au et al., 1983).

The lack of cytoplasm in mature spermatozoa causes deficiency in antioxidant defense system which is compensated by the enzymatic and non-enzymatic antioxidants abundant in seminal fluid. However, the plasma membrane of spermatozoa is rich in PUFA which are susceptible to free radical damage and consequent loss of membrane integrity (Suzuki and Sofikitis, 1999). From our study, L-arginine treatment did not attenuate the lipo-peroxidation following experimental cryptorchidism in rats. MDA levels in groups A and C were significantly high. Decline in antioxidant enzymes such as superoxide dismutase, catalase and glutathione reductase which are markers of oxidative stress (Kehinde et al., 2005) were significantly altered to the negative in cryptorchid rats and treatment with L-arginine did not reverse nor protect the testis from oxidative damage. It is possible that the increased oxidative stress (high MDA levels) may be responsible for the depression of these antioxidant enzymes or alternatively, the inherent decreased expression of these enzymes may be responsible for the increased oxidative stress (Sikka et al., 1995). The result is increased lipid peroxidation, decreased spermatozoa motility, function and ultimately infertility.

**CONCLUSION**

In conclusion, our study demonstrated the detrimental effect of experimental cryptorchidism on testicular...
function and also showed the capacity of L-arginine in augmenting these deleterious effects.

REFERENCES


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