Effect of insulin–like growth factor–1 on apoptosis of rat testicular germ cells induced by testicular torsion

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OBJECTIVE

To investigate the possible protective role of insulin-like growth factor–1 (IGF–1, reported to have a protective effect in experimental models of hypoxic ischaemia), and the involvement of apoptotic cell death in a model of torsion/detorsion of the rat testis.

MATERIALS AND METHODS

Adult male Wistar rats were divided into five groups of five rats each. Group 1 underwent a sham operation as a control; in group 2 the testis was twisted and in group 3 then untwisted; in group 4 IGF–1 was injected subcutaneously just before bilateral torsion, and then the right testis removed after 4 h and the left after 24 h; in group 5, IGF–1 was injected immediately after bilateral detorsion and then the testes removed as in group 4. Both testes were examined histologically, with apoptosis detected using the in situ DNA fragmentation (TUNEL) system, with combined enzymology and immunohistochemistry techniques.

RESULTS

In groups 2 (torsion) and 3 (detorsion), light microscopy of the testis showed some degenerative changes in the germ cells. Compared to group 1, apoptosis was more significant in group 3 than in the other groups. Group 4 (torsion/IGF–1) had a similar number of apoptotic germ cells as in group 2 (torsion) after 24 h, but fewer than the same group after 4 h. In group 5 (detorsion/IGF–1), apoptosis was reduced by IGF–1 significantly more than in group 3 (P < 0.05). Apoptosis was significantly less in spermatids in group 5 than in group 3 (P < 0.05).

CONCLUSIONS

IGF–1 seems to lower the levels of germ cell apoptosis, which may be important for protecting the testes from torsion/detorsion injury. Further clinical studies are needed to evaluate this protective effect in testicular torsion/detorsion.

KEYWORDS

IGF–1, apoptosis, torsion, testes, rat

INTRODUCTION

Testicular torsion leads to tissue degeneration and usually requires emergency surgical intervention to allow reperfusion of the affected tissue [1]. The main pathophysiology of testicular torsion is ischaemia–reperfusion (I–R) injury of the testis caused by the twisted spermatic cord and its release [2]. The ischaemic injury of the ipsilateral twisted testis, termed ‘necrotic’, is followed by the loss of its endocrine and exocrine functions [3]. Although necrotic cell death has been thought to be the predominant type of cell death after I–R of the testis, evidence has shown the involvement of apoptotic cell death. A 3-h, 720° testicular torsion resulted in three times the number of apoptotic germ cells per cross-sectional area than in sham-operated testes; there was a detectable increase in apoptosis 4 h after repairing the torsion [4,5].

IGF–1 and IGF–2 are locally produced in the testis and may regulate the balance between pro- and anti-apoptotic proteins at a cellular level. Activation of the IGF–1 receptor can protect against apoptosis through two alternative pathways, one involving the activation of mitogen-activated protein kinase, and another that results in the mitochondrial translocation of Raf [6]. The objectives of the present study were to determine whether IGF–1 could lower apoptosis and to increase tissue survival after testicular ischaemia in a rat model.

MATERIALS AND METHODS

Ethical approval for the study was granted by the University Ethics Committee. Twenty-five adult male Wistar rats (220–260 g) were maintained on a standard diet and water ad libitum in a quiet, temperature and humidity-controlled room (22 ± 3°C; 12/12 h light-dark cycle); they were anaesthetized intraperitoneally with 90 mg/kg ketamine and 10 mg/kg xylazine and randomly separated into five equal groups. Group 1 had a sham operation; group 2 had a 4-h, 720° torsion of the spermatic cord and then the testis was removed; group 3 had the same torsion but the testis was untwisted after 4 h; group 4 had IGF–1 (20 μg/kg, Sigma Chem Co, St. Louis, Mo) injected subcutaneously just before bilateral torsion, the right testis for 4 h and the left for 24 h, with both testes removed immediately after torsion; group 5 had the same treatment but with the testes untwisted before IGF–1 was injected. All removed testicles were fixed in 4% paraformaldehyde and processed for histological study; 5 μm paraffin sections were stained with haematoxylin and eosin (H&E) and in situ apoptosis detected using DNA fragmentation through a combination of enzymology and immunohistochemistry techniques. The enzyme reaction generates an insoluble, coloured precipitate where DNA has fragmented; the sites were revealed using diamino benzidine staining under light microscopy. In addition, diapedesis, leukocytic infiltration, and necrosis were examined in the interstitial connective tissue by H&E staining. The testicles were also evaluated by light microscopy.

For immunocytochemistry, tissues were fixed in 4% paraformaldehyde for 16–18 h at 4°C.
and further post-fixed overnight in the same fixative. To determine whether there was DNA fragmentation characteristic of apoptosis testicular tissue was stained using the in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) technique. Testis sections (5 μm) were deparaffinized and rehydrated, and immunocytochemistry applied using the streptavidin-horseradish peroxidase method (Albio, R&D Systems, Turkey). As a positive control slides were pretreated with DNAse to produce TUNEL-positive staining of all nuclei, and negative controls were incubated with no TdT enzyme. Immunoreactivity was examined by light microscopy.

In each immunostained section the apoptotic cells were counted in 0.0625 mm² (250 × 250 μm²) fields of seminiferous epithelium with an ×40 objective, using an ocular micrometer system (BX50F-3; Olympus, Tokyo, Japan). This semiquantitative procedure allows the different germ cell populations to be compared [7]. The number of apoptotic cells in the total area was taken as a numeric variable and assessed for a normal distribution; the values were not normally distributed and thus nonparametric methods were used for the statistical analysis, i.e. the Wilcoxon rank sum test for differences in means between samples, and the Kruskal–Wallis test for more than two samples.

The rate of apoptosis in different stages of spermatogenesis (primary, secondary spermatocytes and spermatids) was counted in 20 seminiferous tubules of each section and the rate of apoptosis in germ cells analysed as a categorical variable with the Pearson chi-square and Fisher’s exact tests. For all tests \( P < 0.05 \) was taken to indicate significance.

**RESULTS**

In group 1 the seminiferous tubules were composed of Sertoli cells and germ cells in various stages of differentiation. Spermatogenesis occurred in waves along the length of the seminiferous tubules, and thus adjacent areas of the same tubule showed spermatocytogenesis and spermiogenesis at various stages. Apoptotic cells were seen only in primary spermatocytes (Fig. 1a). The quantitative results are shown in Table 1. In group 2, light microscopy showed degenerative changes in the germ cells, i.e. separation, desquamation, vacuolization, germ cell depletion, necrosis and apoptosis (focal loss in seminiferous epithelium and vasodilatation, hyperaemia, haemorrhage in interstitial tissue; Fig. 1b). The twisted testis had more apoptotic germ cells than in group 1 (\( P < 0.05 \); Table 1). Apoptotic cells were seen in primary, secondary spermatocytes and spermatids. In group 3 the results from light microscopy were similar to those in group 2; there was no cell cohesiveness, but disarray, separation of germ cells, necrotic cells, cellular debris (focal loss and leukocytic infiltration) (Fig. 1c). There were 30 times

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**TABLE 1** The mean (±SD) number of apoptotic cells per 10 tubules, and in relation to germ cells in seminiferous tubules of each group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic cells, 4 h</td>
<td>0.50 (0.52)</td>
<td>2.0 (1.5)</td>
<td>15 (4.0)</td>
<td>0.8 (0.6)</td>
<td>8.9 (6.4)</td>
</tr>
<tr>
<td>24 h</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.4 (1.5)</td>
<td>8.9 (4.4)</td>
</tr>
<tr>
<td>Spermatocyte [24 h]</td>
<td>primary</td>
<td>0.25</td>
<td>0.9</td>
<td>0.8 (0.6)</td>
<td>0.5 (0.4)</td>
</tr>
<tr>
<td></td>
<td>secondary</td>
<td>0.25</td>
<td>0.75</td>
<td>0.25 (0.3)</td>
<td>0.5 (0.5)</td>
</tr>
<tr>
<td></td>
<td>Spermatid</td>
<td>0.25</td>
<td>0.75</td>
<td>0.15 (0.3)</td>
<td>0.15 (0.4)</td>
</tr>
</tbody>
</table>
more apoptotic germ cells than in group 1
(P < 0.05; Table 1). Apoptosis was seen in the
different stages of spermatogenetic cells in
group 3 but it was more prominent in group 3
than in group 2.

In group 4 there were fewer degenerative
changes in the germ cells and better tissue
integrity than in groups 2 and 3; the number of
apoptotic germ cells/unit area was not
significantly different from that in group 1
after 4 h of torsion, while after 24 h there was
a fivefold increase (P < 0.05; Table 1).
However, there were significantly fewer
apoptotic germ cells after IGF-1 and 4 h of
torsion but no difference between groups 2
and 5 after 24 h (P < 0.05). Apoptotic bodies
decreased in spermatogenetic cells in the 4 h
torsion in group 4 (Fig. 1d) but increased in
24 h/IGF-1 torsion.

In group 5 there were fewer degenerative
changes and better tissue integrity in the
germ cells than in groups 2 and 3, but
leukocytic infiltration was evident in
interstitial connective tissue. There were
fewer apoptotic bodies in germ cells than in
group 3, and significantly so in spermatids
(P < 0.05; Fig. 1e). There were significantly
more apoptotic germ cells than in group 1
(P < 0.05) but IGF-1 after both 4 h and
24 h of detorsion significantly reduced the
number of apoptotic germ cells compared
with group 3 (P < 0.05). Apoptosis for all
groups for germ cells is also summarized
in Table 1.

DISCUSSION

Acute testicular torsion and repair in the rat
results in germ cell-specific apoptosis with
the retention of functional Leydig and Sertoli
cell populations [8–10]. Apoptosis is a
physiological form of cell death that occurs
in embryonic development and during
involution of organs. It is characterized by
distinct biochemical and morphological
changes such as DNA fragmentation, plasma
membrane blebbing and cell-volume
shrinkage. Hypoxia–ischaemia induces
apoptotic and necrotic cell death, which
results partly from persistent alterations in
cellular energy homeostasis [5]. Germ cell
apoptosis is stimulated contemporaneously
with the margination and diapedesis of
leukocytes, and an increase in intratesticular
oxidative damage, so it has been hypothesized
that the pathology seen after testicular
torsion/detorsion is the result of classical I-R
injury [4]

Apoptosis occurred predominantly in
spermatocytes, early and late spermatids, and
Sertoli cells. In contrast, spermatogonia,
peritubular connective tissue (fibroblasts and
myofibroblasts) and endothelial cells seldom
underwent apoptosis. Leydig cells were
affected less often than spermatocytes [11]. In
the present study all groups other than the
first had apoptosis in primary and secondary
spermatocytes and spermatids, but
significantly fewer apoptotic bodies were
detected in spermatids in group 5. However,
apoptosis was more prominent in group 3
detorsion) than in group 2 (torsion), which
may be related to the time of the I-R injury.
The explanation is possibly that IGF-1
decreases apoptosis in susceptible cells if
the apoptotic process has not begun. So
apoptotic cell death may follow two different
apoptotic pathways, e.g. activation of caspase
8 or caspase 9, which activates the cascade
leading to cell death [12–15]. The activation of
the IGF-1 receptor can inhibit the activation
of different members of caspase family,
through signalling via the phosphoinositol 3-
kinase pathway. Recently, IGF-1 was reported
to block the release of mitochondrial
cytochrome c, which is essential in
orchestrating the sequential activation of
different caspases and that culminates in
apoptosis. Importantly, IGF-1 cannot protect
cells from apoptosis once cytochrome c
enters the cytoplasm, reinforcing an earlier
observation that IGF-1 lowers the probability
of apoptosis occurring in susceptible cells, but
has no effect on the kinetics of apoptosis
once the process is initiated [16].

Many hormones, cytokines and growth
factors are known to act as general and/or
tissue-specific survival factors preventing
the onset of apoptosis. Several studies have
examined the ability of a possible protective
role of IGF-1 in hypoxia–ischaemia in several
organs of the neonatal rat, e.g. brain,
myocardium, kidneys and intestines [17–20].
IGF-1 levels peaked 4 h after its injection,
remaining elevated for 15 h and returning
to basal levels 24 h later [21]. However,
pretreatment with allopurinol, ATP-MgCl2 [2]
or surfactant prevent such reperfusion injury
in animal models [22–24].

In the present study, IGF-1 decreased germ
cell apoptosis in group 4 with 4 h of torsion,
which might be explained by the pathway of
cytochrome c and apoptosis. However,
apoptosis increased 24 h after torsion and
IGF-1 administration, which could be related
to the half-life of IGF-1.

Detorsion alone does not seem to be enough
to protect from classical I-R injury, but
apoptosis was significantly less in group 5,
and thus we think that classical I-R injury
related to apoptosis may be prevented by IGF-
1 then detorsion in susceptible cells, even
though the treatment is given after 4 h of
torsion. The findings in group 5 at 24 h were
similar to those in group 4 at 4 h but different
from those in group 4 at 24 h. This difference
is possibly related to tissue reperfusion,
although the serum IGF-1 level returned to
normal after 24 h. Finally, we think that IGF-1
after detorsion may increase the chance of a
positive result in acute testicular torsion, even
after 4 h of torsion.

To our knowledge, this is the first report of the
effect of IGF-1 on apoptosis in testicular
torsion; the results show that IGF-1
significantly reduced the level of germ cell
apoptosis after classical I-R injury. If this
theory is supported by further clinical studies,
IGF-1 may be administered in cases of acute
testicular torsion to increase tissue survival.

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CONFLICT OF INTEREST

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**Abbreviations:** I-R, ischaemia-reperfusion; H&E, haematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling.