Reproductive biology

Protective effect of curcumin in cisplatin-induced oxidative injury in rat testis: mitogen-activated protein kinase and nuclear factor-kappa B signaling pathways

Yusuf Ozlem Ilbey1,4, Emin Ozbek1, Mustafa Cekmen2, Abdulmuttalip Simsek1, Alper Otunctemur1, and Adnan Somay3

1Department of Urology, Bezm-i Alem Valide Sultan Valif Gureba Research and Education Hospital, Aksaray, Istanbul, Turkey 2Department of Biochemistry, Kocaeli University, Kocaeli, Turkey 3Department of Pathology, Bezm-i Alem Valide Sultan Valif Gureba Research and Education Hospital, Istanbul, Turkey 4Correspondence address. Tel: +90-212-534-69-70; Fax: +90-212-621-75-80; E-mail: ozlemyusufilbey@hotmail.com

Background: The aim of this study was to investigate the cellular/biochemical mechanisms by which cisplatin (CIS) causes testicular toxicity. We evaluated the role of inducible nitric oxide synthase (iNOS) expression, mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-kB) activation in the pathogenesis of testicular damage induced by CIS, and investigated the effects of curcumin (CMN) against CIS-induced testicular injury in rats.

Methods: Rats were divided into five equal groups: (1) control, (2) CIS, (3) CMN, (4) CIS + CMN and (5) CIS + corn oil. After the treatment, body and testicular weights, and plasma testosterone levels were observed, along with the biochemical, histopathological and immunohistochemical changes in testes.

Results: Testicular weight, plasma testosterone levels, activities of glutathione peroxidase (GSH-Px) and glutathione (GSH) levels significantly decreased, whereas the level of malondialdehyde (MDA) and nitric oxide (NO) significantly increased with CIS compared with the controls. A significant increase in plasma testosterone levels, GSH levels and GSH-Px activity, and a decrease in MDA and NO levels in testicular tissue were observed with CIS + CMN compared with that with CIS alone. There was marked staining for iNOS, MAPK/p38 and NF-kB/p65 expression with CIS compared with the control and CIS + CMN groups. CIS caused irregular seminiferous tubules, reduction of seminiferous epithelial layers, significant maturation arrest and perivascular fibrosis. CMN administration to CIS-treated rats significantly prevented these histopathologic changes.

Conclusions: MAPK and NF-kB activation have a significant role in CIS-induced testicular toxicity. CMN has a strong potential for use as a therapeutic adjuvant in CIS gonadotoxicity.

Key words: curcumin / nitric oxide / NF-kB / cisplatin / testicular damage

Introduction

Cisplatin (CIS) is a highly effective antineoplastic DNA-alkylating agent used to treat many types of various solid tumors testicular, bladder, ovary, cervix, endometrium, lung, head and neck (Colpi et al., 2004; Howell and Shalet, 2005). However, effective anticancer therapy with this cytotoxic drug is limited by its reproductive toxicity, as has been documented in the various experimental studies (Cherry et al., 2004).

The mechanism underlying CIS’s anticancer activity is incompletely defined, but it is generally accepted that CIS is a DNA-damaging agent which forms CIS–DNA adducts that kill cells via several mechanisms, resulting in the induction of apoptosis (Wang and Lippard, 2005). Reactive oxygen species (ROS) is a recently recognized mechanism in the pathogenesis of the CIS-induced testicular toxicity in experimental studies (Atesşahin et al., 2006; Türk et al., 2008). CIS causes lipid peroxidation (LPO) and decreases the activity of enzymes that protect against oxidative damage in testicular tissue from CIS-treated rats.
Numerous studies have shown that CIS exposure disrupts the redox balance of tissues, suggesting that biochemical and physiological disturbances result from oxidative stress (Antunes et al., 2001; Silva et al., 2001). Yamaguchi et al. (2008) have reported that several signaling pathways may be involved in modulating cell survival or apoptosis in response to CIS-induced DNA damage, and these signaling pathways can also be activated by oxidative stress and LPO (Ghosh et al., 1998; Widmann et al., 1999).

Nitric oxide (NO) is a free radical that is largely synthesized by the enzyme NO synthase (NOS). Of the three NOS isoforms, two are constitutively expressed, endothelial NOS and neuronal NOS, and one, inducible NOS (iNOS), is regulated at the gene level by a variety of mediators (Bredt, 1997; MacMicking et al., 1997). Since iNOS is responsible for the production of sustained high levels of NO, it is often considered the primary perpetrator of autotoxicity under oxidative stress (Bogdan et al., 1998). A large body of pharmacological and genetic evidence has demonstrated that NO, together with ROS, is essential for triggering cell death (Zaninotto et al., 2006). Oxidation products of NO can induce LPO, S-nitrosylation of thiol groups in proteins and inhibition of enzymes for mitochondrial respiration (Jiang et al., 2006). Under the simultaneous generation of NO and ROS, the cellular antioxidant capabilities are also suppressed (de Pinto et al., 2002). In addition, it has been demonstrated that NO may enhance cellular injury by decreasing intracellular glutathione (GSH) levels (Zhang et al., 2000).

GSH is one of the most important molecules in the cellular defense against chemically reactive toxic compounds or oxidative stress (Yu and Anderson, 1997). Decreased cellular GSH levels and a decreased capacity for GSH synthesis sensitize cells to certain drugs. GSH synthesis is induced in cells exposed to oxidative stress as an adaptive process. Therefore, interest has been focused in compounds that act as antioxidants and are capable of stimulating GSH synthesis.

The process of iNOS expression involves different signal transduction pathways, including nuclear translocation of the transcription factor nuclear factor-kappa B (NF-kB) (Gilad et al., 1998). NF-kB belongs to the Rel family of transcriptional activator proteins and is present in the cytoplasm in an inactive state, bound with the inhibitory IkB subunit proteins. NF-kB is activated by a variety of external stimuli, including oxidative stress, that demonstrate significant testicular toxicity in rats (Ates¸s¸ahin et al., 2001; Silva et al., 2001). CIS was injected intraperitoneally (i.p.) at the single dose of 7 mg/kg. The dose of CIS was selected according to previous studies (24 h light/dark cycle, 26–28°C) for at least 1 week before the experiment and those conditions were preserved until the end of the experiment. Animal cages were kept clean, and food and water were given regularly every day. All experiments in this study were performed in accordance with the guidelines for animal research issued by the National Institutes of Health and were approved by the Local Committee on Animal Research.

Study design and treatment
The rats were randomly divided into five groups, all of which contained six animals. CIS was injected intraperitoneally (i.p.) at the single dose of 7 mg/kg. The dose of CIS was selected according to previous studies that demonstrated significant testicular toxicity in rats (Ates¸s¸ahin et al., 2006; Türk et al., 2008). CMN was dissolved in corn oil and given orally at the dose of 200 mg/kg/day. The dose of CMN was selected based on the results of recent studies where the antioxidant and anti-inflammatory action of this agent was apparent (Chuang et al., 2000; Bayrak et al., 2008), and it was given for 10 consecutive days after a single i.p. dose of CIS. Corn oil was the vehicle of CMN, and it was also given for 10 consecutive days after a single i.p. dose of CIS. Group 1 served as control and received a single dose i.p. injection of 1 ml of distilled water. Group 2 rats were treated with CIS alone. Group 3 received CMN alone. Rats in Group 4 received CIS + CMN. Group 5 rats received CIS + corn oil.

Sample collection
The rats were weighed and they all were sacrificed under anesthesia (Ketamin 200 mg/kg body weight, i.p.) at the end of 10 days. Abdomen
was reached with an abdominal midline incision and orchietomy was performed eventually. After weighing testes, one of the testes was stored at −80°C for biochemical evaluation. The other testis was fixed with Bouin’s solution for histopathologic examination. Blood samples were collected from aorta and separated into plasma for biochemical examinations.

Biochemical studies

Plasma testosterone level

The plasma testosterone level was examined to evaluate chemotherapy-associated hypoandrogenism and was measured with the immunoenzymatic method according to the protocol described by Srivastava (2001).

LPO level

Testicular tissue was removed and homogenized in a Teflon-glass homogenizer with a buffer containing 1.5% potassium chloride to obtain 1:10 (w/v) whole homogenate. Malondialdehyde (MDA), which formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA, referred to as thiobarbituric acid reactive substance, was measured with thiobarbituric acid at 532 nm in a spectrophotometer, as described previously (Wasowicz et al., 1993). The MDA level was expressed as nmol/g wet tissue.

Glutathione peroxidase activity

Glutathione peroxidase (GSH-Px) activity was measured according to Paglia and Valentine (1967), by monitoring the oxidation of reduced NADPH at 340 nm. Enzyme units were defined as the number of micromoles of NADPH oxidized per minute and calculated using the extinction coefficient of NADPH at 340 nm (6.22 × 10⁵/mole/cm). Results were reported as units per gram protein.

GSH level

Reduced GSH was estimated by the method of Moron et al. (1979), where the color developed was read at 412 nm. Protein concentrations in all samples were measured using the method of Lowry et al. (1951). Results were reported as µmol/g wet tissue.

NO level

Total nitrite (NO₂⁻) was quantified by the Griess reaction (Granger et al., 1999), after incubating the supernatant with Escherichia coli nitrate reductase to convert NO₂⁻ to NO₃⁻. Griess reagent (1 ml of 1% sulfanilamide, 0.1% naphthyl-ethylenediamine hydrochloride and 2.5% phosphoric acid; Sigma Chemical Co.) was then added to 1 ml of supernatant. The absorbance was read at 545 nm after a 30 min of incubation. The absorbance was compared with the standard graph of NaNO₃, obtained from the reduction of NaNO₂ (1–100 µmol/l). The accuracy of the assay was checked in two ways; the inter- and intra-assay coefficients of variation were 7.52% and 4.61%, respectively. To check conversion of nitrate to nitrite (recovery rate), predetermined amounts of nitrate were added to control plasma samples; these samples were deproteinized and reduced as above. The results were expressed as nmol/g wet tissue.

Histopathologic examination

Evaluation of testicular spermatogenesis

Testicular spermatogenesis was evaluated by measuring testicular weight, mean seminiferous tubular diameter and thickness of germ cell layers. Testis was excised and weighed. Testicular tissue (~5–10 mg) was prepared for histologic examination. Semi-thin paraffin wax testicular tissue sections (4 µm thick) fixed in Bouin’s solutions were stained with hematoxylin and eosin and were examined under a light microscope under ×400 magnification using standard techniques (Olympus, Tokyo, Japan).

Evaluation of testicular fibrosis

In order to evaluate testicular fibrosis, specimens obtained from testis were embedded in paraffin, sectioned at 4 µm and stained with Masson’s trichrome. Specimens were scored briefly after painting as follows: (−) no fibrosis, (+) fibrosis in fewer than 25% of total testicular tissue (mild), (++) fibrosis in 25–50% of total testicular tissue (moderate), (+++) fibrosis in over 50% of total testicular tissue (serious) (Ayvildiz et al., 2004).

Immunohistochemical studies

For immunohistochemical evaluation, specimens were processed for light microscopy and sections were incubated at 60°C overnight and then de-waxed in xylene for 30 min. After rehydrating in a decreasing series of ethanol, sections were washed with distilled water and PBS for 10 min. Sections were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37°C for 15 min and washed with PBS.

Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H₂O₂ for 15 min to inhibit endogenous peroxidase activity. Then, sections were incubated with NF-kB/p65 (Rel A) Ab-1 (R-B-1638-R7, Neomarkers, Labvision, Fremont, CA, USA), MAPK/p38 (Vector Laboratories, Burlingame, CA, USA) and iNOS Ab-1 (R-B-1605-R7, Neomarkers, Labvision, Fremont, CA, USA) antibodies. The Ultra-vision (Labvision) horseradish peroxidase/3-amino-9-ethylcarbazole staining protocol was used at this stage.

Sections prepared for each case were examined by light microscopy. Sections of rat lung were used as the control for immunohistochemical staining specificity, according to data provided by the antibody manufacturer.

According to the diffuseness of the staining, sections were graded as: 0, no staining; 1, staining <25%; 2, staining between 25% and 50%; 3, staining between 50% and 75%; or 4, staining >75%. According to staining intensity, sections were graded as: 0, no staining; 1, weak but detectable staining; 2, distinct staining; and 3, intense staining. Immunohistochemical values were obtained by adding the diffuseness and intensity scores.

Statistical analyses

Statistical analyses of the histopathologic and immunohistochemical evaluation of the groups were carried out by the χ² test and analyses of the biochemical data were by the Mann–Whitney U test. Results of all groups are shown as mean values ± standard deviation. All results were compared one by one with other groups’ results and with control group results. A value of P < 0.05 was accepted as statistically significant.

Results

The biochemical, histopathological and immunohistochemical results were similar for the control (1) and CMN (3) groups, and for the CIS (2) and CIS + vehicle (5) groups. We therefore decided to consider them without distinction and report only the control and CIS groups.

Body and testicular weight

Although significant testicular weight loss was observed in the group that received CIS only compared with the control group (P < 0.01),
there was no marked reduction in body weight between these groups (P > 0.05). Co-administration of CMN in CIS-treated rats preserved the testicular weight (P < 0.05) (Fig. 1A).

Biochemical variables in plasma and tissue

Plasma testosterone levels decreased significantly in rats treated with CIS alone compared with the control group (P < 0.001); but co-administration of CMN in CIS-treated rats significantly increased the plasma testosterone level (P < 0.001) compared with CIS alone (Fig. 1D).

The GSH-Px activity and GSH levels in testicular tissue of rats treated with CIS alone were significantly lower than those in control group (P < 0.01), while treatment with CMN in conjunction with CIS significantly elevated the GSH-Px activity and GSH levels (P < 0.01) (Table I).

The MDA and NO levels in the testicular tissue were found to be significantly higher in rats treated with CIS alone than those in the control group (P < 0.01), and treatment with CMN prevented the elevation of MDA and NO levels in CIS-treated rats (P < 0.001) (Table I).

Histologic examination

Spermatogenesis

Control rats showed normal testicular morphology with regular spermatogenesis and normal Sertoli cells (Fig. 2A). The diameter of the seminiferous tubules and germinal cell thickness after CIS were smaller than the controls (Fig. 1B and C). All rats treated with CIS alone were characterized by a depletion of germ cells, irregular seminiferous tubules exhibiting Sertoli cell-only type and a few spermatagonia. Reduced seminiferous epithelial layers were found in numerous tubules, and irregular and diminished tubules containing a few germ cells were also seen (Fig. 2B). Significant maturation arrest was also observed in the group which received CIS alone compared with the control group (P < 0.001). Spermatogenesis was significantly preserved in the rats treated with CIS + CMN; the morphological characteristics of these testes were comparable to those in control groups (Fig. 2C).

Testicular fibrosis

Perivascular fibrosis and hyalinization of intertubular connective tissue were not observed in the control group and in the group that received CMN + CIS. In the rats receiving only CIS, mild perivascular fibrosis and hyalinization of intertubular tissue were observed.

Immunohistochemical studies

On immunohistochemical evaluation, there was more intense expression of iNOS, p38-MAPK and p65-NF-kB in rats subjected to CIS alone compared with control (P < 0.01) (Fig. 3A–I, Table II). There was poor or slight expression of iNOS, p38-MAPK and p65-NF-kB in the control and CIS + CMN groups, and there was no significant difference between these two groups (P > 0.05).

Discussion

Oxidative damage caused by ROS has been implicated in the pathogenesis of CIS-induced testicular injury. In the present study, testicular...
Curcumin prevents testicular toxicity

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>CIS</th>
<th>CMN</th>
<th>CIS + CMN</th>
<th>CIS + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-Px</td>
<td>9.26 ± 0.51</td>
<td>5.01 ± 0.5***</td>
<td>8.82 ± 0.43</td>
<td>9.2 ± 0.46***</td>
<td>5.48 ± 0.35</td>
</tr>
<tr>
<td>GSH</td>
<td>1.48 ± 0.11</td>
<td>1.01 ± 0.03***</td>
<td>1.49 ± 0.07</td>
<td>1.69 ± 0.23***</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td>MDA</td>
<td>40.4 ± 1.59</td>
<td>66.8 ± 6.71***</td>
<td>39.8 ± 0.99</td>
<td>32.6 ± 6.75***</td>
<td>68.2 ± 5.84</td>
</tr>
<tr>
<td>NO</td>
<td>46.5 ± 1.65</td>
<td>95.5 ± 4.48***</td>
<td>45.8 ± 2.46</td>
<td>48.2 ± 1.35***</td>
<td>93.3 ± 3.21</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six rats in each group. GSH-Px, glutathione peroxidase, nmol/g wet tissue; GSH, reduced glutathione, μg wet tissue; MDA, malondialdehyde, nmol/g wet tissue; NO, nitric oxide, nmol/g wet tissue.

Groups: control, cisplatin (CIS), curcumin (CMN). CIS + CMN and CIS + vehicle.

'α' compared with control group and 'β' compared with CIS group.

Control group compared with CMN, CIS + CMN group and CIS + vehicle group compared with CIS group and P > 0.05.

***P < 0.01.

parenchymal atrophy and histopathologic examination showed severe degeneration, necrosis, reductions in seminiferous tubules alongside reduction in germinal cell thickness in the testes of rats treated with CIS alone, the effects of this agent on testes may be due to its specific toxic effects on the target organ and not due to its general toxicity. In the present study, CMN treatment prevented a reduction in testicular weight in the CIS + CMN group of rats when compared with those in the CIS alone group. This protective effect of CMN can be explained by the fact that it prevents cellular damage occurring as a result of oxidative stress in spermatogenic cells of seminiferous tubules and Leydig cells of the stroma.

In the current study, plasma testosterone levels were also significantly lowered in CIS-treated rats. The decreased plasma testosterone level in CIS-treated rats could be attributed to the impairment of Leydig cells. It has also been reported that the CIS-induced changes in testosterone are associated with decreased numbers of LH receptors on Leydig cells (Maines et al., 1990). The administration of CMN to CIS-treated rats significantly increased the testosterone level. The spermatogenic inhibition in CIS-treated rats indicated in the present study cannot only be the result of the reduced plasma testosterone level. Besides hormonal alteration, the spermatogenic inhibition may also be due to the formation of free radical products in the testicular tissue as they exert a detrimental effect on spermatogenesis. The improvement that observed in spermatogenesis among CMN-delivered rats may be associated with the antioxidant and free radical scavenger properties of the CMN.

Oxidative stress and activation of MAPKs are known to stimulate transcription factors, including NF-kB (Bowie and O’Neill, 2000; Kyriakis and Avruch, 2001; Bubici et al., 2006). NF-kB is known to activate many genes, including iNOS, resulting in excessive NO generation (Xie et al., 1994; Kleniert et al., 2004). Excessive production of NO causes vasodilatation and hypotension leading to organ hypoperfusion, edema and organ dysfunction. NO can interact with ROS to form peroxynitrite, which is a powerful oxidant and cytotoxic agent and may play an important role in the cellular damage associated with the overproduction of NO. It is generally thought that the endothelial NOS-derived NO, at low levels, regulates the physiological vasodilatation, whereas excessive NO production due to elevated expression of iNOS can cause cytotoxic effects on surrounding cells. The contribution of NO to tissue damage can be a direct effect mediated by NO itself (Davis et al., 2001) and an indirect effect mediated by...
Figure 3 Immunohistochemical staining showing iNOS, p38/MAPK and p65/NF-κB expression.

(A) Focal mild staining (score 2) with iNOS in control (×200). (B) Diffuse iNOS staining (score 7) in CIS group (×200) (arrows). (C) Mild iNOS staining (score 3) in CIS + CMN (×200) (arrows). (D) Low MAPK/p38 positivity (score 2) in control (×200). (E) Diffuse, intensive MAPK/p38 positivity (score 7) in CIS group (×200) (arrows). (F) Low MAPK/p38 positivity (score 3) in CIS + CMN (×200) (arrows). (G) Low NFκB/p65 positivity (score 2) in control (×200). (H) Diffuse, intensive NFκB/p65 positivity (score 7) in CIS group (×200) (arrows). (I) Low NFκB/p65 positivity (score 3) in CIS + CMN (×200) (arrows).
reactive nitrogen species produced by the interaction of NO with superoxide anions or oxygen (Wink and Mitchell, 1998; Davis et al., 1999). In the present study, CMN treatment reduced the increase of iNOS expression in the testicular tissue of CIS-treated rats; this effect probably results from the reduction of NF-kB expression. The mechanism by which CMN affects the NF-kB and iNOS pathways remains to be determined. However, probably this effect of CMN stems from its decreasing impact on ROS levels and inhibitive influence on p38-MAPK activation (Camacho-Barquero et al., 2007).

It has been reported that inhibition of active NF-kB by dexamethasone, acetyl salicylic acid or pyrrolidine dithiocarbamate was caused by suppression of only the p65 subunit of NF-kB (Nakashima et al., 1999), and the p50 subunit was not inhibited by the NF-kB inhibitors. Therefore, in this study, we only examined the p65 subunit by immunohistochemistry.

The p38-MAPK pathway is increasingly recognized as an important molecular component activated in response to a variety of stresses including oxidative stress (Chang and Karin, 2001), which is widely considered to play an important role in the development of CIS-induced testicular toxicity. In many human cancer cell lines, CIS preferentially activates p38-MAPK compared with the other members of MAPK family. Therefore, we studied only p38-MAPK immunohistochemically. In the present study, the immunohistochemical observations showed a significantly increased number of p38-MAPK-positive cells in rats treated only with CIS when compared with controls. This expression was also reduced by treatment with CMN.

Reverse transcriptase–polymerase chain reaction or western blotting analyses are functional assays by which the actual activity of iNOS, p38-MAPK and NF-kB/p65 can be measured. Western blotting provides a more quantifiable way of measuring iNOS, p38-MAPK and NF-kB/p65 subunit activities. Therefore, the absence of western blotting analyses may be a limitation of this study. However, similar to our study, many studies in the literature have determined p38-MAPK, NF-kB/p65 and iNOS expression by immunohistochemical staining method (Chatterjee et al., 2001; Jang et al., 2006; Notebaert et al., 2008; Pourazar et al., 2008).

Germ cell apoptosis has been reported to play an important role in CIS-induced testicular damages (Zhang et al., 2001; Cherry et al., 2004). The lack of investigation on whether CMN has affected the apoptotic properties of CIS may be a limitation of this study. However, in a recent study, ginger and roselle, which are antioxidant and anti-inflammatory agents like CMN, have been reported to prevent CIS-mediated apoptotic cell death and reduce CIS-related testicular damage in testicular tissue and sperm cells (Amin, et al., 2008).

We believe that CMN may prevent apoptosis by a similar mechanism and prevent testicular damage, as well.

In the present study, co-administration of CMN with CIS prevented the damage to testis induced by this drug. However, CMN also may decrease the efficacy of chemotherapy based on CIS. Another experimental study may be designed to evaluate whether CMN decreases the efficacy of anti-tumor chemotherapy in a tumor model.

In conclusion, the findings of the present study reinforce the significant role of ROS and show that MAPK and NF-kB activation and iNOS expression also play a key role in the pathogenesis of CIS-induced oxidative injury in the rat testis. The present study also demonstrates that blockade of NF-kB activation by an antioxidant such as CMN could be an effective strategy for prophylaxis of CIS-induced testicular damage. CMN has a potent protective effect against the testicular toxicity of this agent and might be clinically useful. However, further studies are required on this issue before clinical application can be recommended.

**Table II The immunohistochemical staining score in control, CIS, CMN, CIS + CMN and CIS + vehicle groups**

<table>
<thead>
<tr>
<th>Score</th>
<th>Control</th>
<th>CIS</th>
<th>CMN</th>
<th>CIS + CMN</th>
<th>CIS + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>p65</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>p38</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

According to the diffuseness of the staining, sections were graded as: 0, no staining; 1, weak but detectable staining; 2, distinct; 3, intense staining. Immunohistochemical values were obtained by adding the diffuseness and intensity scores.

---

According to staining intensity, sections were graded as: 0, no staining; 1, staining <25%; 2, staining between 25% and 50%; 3, staining between 50% and 75%; or 4, staining >75%. According to staining intensity, sections were graded as: 0, no staining; 1, weak but detectable staining; 2, distinct; 3, intense staining.

References


Submitted on July 24, 2008; resubmitted on February 10, 2009; accepted on February 12, 2009.