**ABSTRACT**

Apoptosis, as well as necrosis, has an important role in post-ischemic renal pathology. The effect of pretreatment with Docosahexaenoic acid+Eicosapentaenoic acid (DHA+EPA) on renal injury and apoptotic protein expression was evaluated. Right nephrectomy was completed on male Wistar rats (255–300 g). The rats received DHA+EPA (200 mg/kg/day) of distilled water orally for 14 days before ischemia reperfusion (IR) or sham operation. A total of 81 rats were divided into three main groups with 6, 24 and 48 h of post-operation or reperfusion period. Serum creatinine (SCr), BUN, creatinine clearance (CCr) and fractional excretion of sodium (FE\textsubscript{Na}) were measured. Tissue levels of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) activities, Bax and Bcl-2 protein expressions and renal histological injury were determined. SCr, BUN and FE\textsubscript{Na} increased 6–48 h of reperfusion (\(P < 0.01\)). Tissue MDA content and Bax expression increased (\(P < 0.01\)) and CAT and SOD activities decreased (\(P < 0.05\)) in the IR group. DHA+EPA decreased SCr and BUN, FE\textsubscript{Na}, tissue MDA levels (\(P < 0.05\) vs. IR) and increased CAT and SOD activities and Bcl-2 expression (\(P < 0.05\) vs. IR) for 6–48 h after ischemia. IR induced mild (6 h, \(P < 0.05\)) and severe (24–48 h, \(P < 0.01\)) tissue damage. Mild-to-moderate tissue damage was observed in DHA+EPA groups from 6 to 48 h of reperfusion period (\(P < 0.05\) vs. IR, 24–48 h). In conclusion, the results suggest that pre-ischemic exposure to DHA+EPA could improve the outcome of early graft function by inhibition of IR-induced oxidative stress and apoptosis.
and mortality [5–7]. Apoptosis results from complex interactions among the Bcl-2 protein family that include anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax [8]. In hypoxic renal cells, especially proximal tubules, Bax is translocated from cytosol to the mitochondrial membrane which causes pathological tissue destruction [9]. It seems that enough knowledge about critical time of renal cell apoptosis during reperfusion will be helpful in improving the outcome of ARF [5].

Previously, strategies have been applied to prevent IR-induced apoptosis in different tissues, including defective herpes simplex viral (HSV) vectors to induce Bcl-2 over-expression in the brain [10–12] and recently Adenovirus-mediated Bcl-2 and Bcl-xl gene transferred in renal tissue [13,14]. However, it is also available through application of pharmacological agents which augment anti-apoptotic proteins and decrease pro-apoptotic proteins [15,16].

In a recent study, we showed that dietary supplementation with n-3 polyunsaturated fatty acids [docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)] decreased IR-induced functional and histological injury in the hippocampus through induction of Bcl-2 and suppression of Bax proteins that last for 10 days after ischemia [17]. Studies on renal IR model also demonstrated that 4-week oral supplementation with EPA in rats improved glomerular filtration rate (GFR) [18], and intraperitoneal injection of DHA decreased SCR, improved histological damage and survival of the rats during the 8 days after ischemia [19]. For this study, we evaluated the effect of 2-week supplementation with DHA+EPA prior to IR induction in the left kidney on renal function and histology and expression of Bcl-2 and Bax proteins at different times post-reperfusion.

**METHODS AND MATERIALS**

**Animals**

Male Wistar rats (255–300 g) with free access to water and a standard commercial diet with fixed value of carbohydrate, protein, fat, fiber and vitamins plus minerals (660, 230, 40, 60 and 10 mg/kg, respectively; Nuvital Nutrients, Curitiba, Parana, Brazil) were used in this study. All experimental procedures were approved by the Ethics Review Committee for Animal Experimentation of the University and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Drugs**

Docosahexaenoic acid+Eicosapentaenoic acid (DHA ethyl ester >98%; 23306 and EPA ethyl ester >96.5%; 23330, Maxomega™, Equateq, London, UK) and for surgical procedures ketamine and xylazine (Alfasan Chemical Co, Woerden, The Netherland) were used. DHA+EPA (200 mg/kg; DHA: 80 mg/kg/day + EPA: 120 mg/kg) was gavaged every day in DHA+EPA-treated groups.

**Experimental design**

The right kidney was removed under general anesthesia with Ketamine hydrochloride 10% (50 mg/kg; i.p.) plus Xylazine 2% (4 mg/kg; i.p.). After one week of recovery period, 81 rats were divided into nine groups each including nine rats (*Table I*), then they received DHA+EPA or distilled water for 2 weeks, after which IR surgery or Sham operation took place.

The method of IR Injury has been discussed previously [20,21], briefly the left renal artery was exposed through a small flank incision and occluded with a non-traumatic arterial clamp. After 45 min of ischemia, the clamp was released, and each animal was stored in a metabolic cage for 6, 24 or 48 h of reperfusion time (depending on experimental group, *Table I*) to record urine volume. Urine samples were stored at −20 °C until assay for creatinine and sodium concentrations. At the end of reperfusion period, the animals were anesthetized, decapitated and blood samples were drawn from the carotid artery and were centrifuged at 2000 *g* for 10 min to determine BUN, serum creatinine and sodium levels.

At the end of study, the abdomen was dissected, and the left kidney was cut and divided longitudinally into two sections; one part stored at −80 °C and used for biochemical analysis and Western blotting to detect protein expression, and the other part fixed and used for histological evaluation.

**Biochemical analysis (SOD and catalase activity and MDA tissue contents)**

Kidney sections that had been prepared for biochemical analysis were homogenized in a homogenization buffer (12.5 mM sodium phosphate buffer pH 7.0, 400 mM NaCl), followed by centrifugation at 1000 *g* for 10 min at 4 °C. The resulted supernatant underwent biochemical analysis.

The method of thiobarbituric acid was used to determine Malondialdehyde (MDA) as an index of lipid peroxidation level in renal tissue [22]. In this method, the
reaction of MDA with thiobarbituric acid (TBA Sigma, St. Louis, MO, USA) produces a red color with the highest absorbance at 532 nm. Results were expressed as nmol MDA per gram of wet tissue (Malondialdehyde Assay kit; NWLSS, Vancouver, WA, USA).

Catalase (CAT) and Superoxide Dismutase (SOD) activities were determined using the commercial kits (ab83464 and ab65354, respectively, CB4 0FL; Abcam Plc, Cambridge, UK) [4]. CAT activity was measured by a spectrophotometric assay of hydrogen peroxide based on the formation of its yellow stable complex with ammonium molybdate. SOD Activity Assay Kit utilizes WST-1, which produces a water-soluble dye upon reaction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. The part of kidney sections that had been prepared for Western blotting (Bcl-2 and Bax expression) were stained with hematoxylin and eosin (H&E). All histological evaluations were fixed in 10% formalin and embedded in paraffin wax, cut at (4–5 μm) and stained with hematoxylin and eosin (H&E). All histological evaluations were done under an optical microscope. Three sections were prepared from each sample and then were evaluated by two pathologists unaware of the experimental groups and protocols. The method for determining the grade of damage to the renal tissue has been described previously [23]. Briefly, it was graded from 1 to 4 according to the following criteria: (0) for no sign of necrosis, (1) for necrosis of individual cells, (2) for necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding

Table 1 Experimental groups and protocol of study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Surgery (45 min)</th>
<th>Post-surgery (h)</th>
<th>Number (%) of each grade observations (percent of each grade observation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sham</td>
<td>Sham operation</td>
<td>Number of slices from each kidney scored by two pathologists. Total observation – number of rats in each group × number of slices from each kidney × two pathologist.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-surgery (6)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>IR*</td>
<td>Ischemia</td>
<td>7 (7.8)</td>
</tr>
<tr>
<td></td>
<td>DHA+EPA+IR*</td>
<td>Ischemia</td>
<td>9 (10.0)</td>
</tr>
<tr>
<td>II</td>
<td>Sham</td>
<td>Sham operation</td>
<td>86 (95.6)</td>
</tr>
<tr>
<td></td>
<td>IR**</td>
<td>Ischemia</td>
<td>6 (6.7)</td>
</tr>
<tr>
<td></td>
<td>DHA+EPA +IR**</td>
<td>Ischemia</td>
<td>10 (11.1)</td>
</tr>
<tr>
<td>III</td>
<td>Sham</td>
<td>Sham operation</td>
<td>80 (88.9)</td>
</tr>
<tr>
<td></td>
<td>IR**</td>
<td>Ischemia</td>
<td>12 (13.3)</td>
</tr>
<tr>
<td></td>
<td>DHA+EPA +IR**</td>
<td>Ischemia</td>
<td>14 (15.6)</td>
</tr>
</tbody>
</table>

**Gavages:** distilled water or DHA+EPA 200 mg/kg/day (1 mL) gavage for 2 weeks before surgery. Number of rats in each group was 9.

Number of each grade observations (percent of each grade observation) in each group is shown. Five slices prepared from each kidney scored by two pathologists unaware pathologists; Total observation = number of rats in each group × number of slices from each kidney × two pathologist.

Mann–Whitney U-test results: *P < 0.05 and **P < 0.01 vs. Sham group with same time of post-operation period, †P < 0.05 vs. IR group with same time of reperfusion period. &P < 0.05 vs. DHA+EPA+IR group with 6 h of reperfusion period.

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tubules, (3) for necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex and (4) for necrosis affecting all the three segments of the proximal convoluted tubule.

Data analysis
As the data showed a normal distribution pattern using a Kolmogorov–Smirnov test, as well as homogeneity of variance, group comparisons were carried out by two-way analysis of variance (2-way repeated ANOVA: between: groups and within: times 6, 24 and 48 h of reperfusion). Further analysis for individual between-group comparisons was carried out with the post hoc Tukey’s test. All data were expressed as mean ± standard error of mean (SEM). In all comparisons, statistical significance levels were determined as $P < 0.05$.

RESULTS
Four rats died during the ischemia or reperfusion period, which were replaced to make a total of nine rats for each group. Their body weight was measured at the beginning of gavage and also at the end of the study before killing the animals. The body weight means for DHA+EPA (200 mg/kg/day) in groups 6, 24 and 48 h reperfusion at the beginning of study were 273.42 ± 6.38 mg, 269.44 ± 9.21 mg and 267.11 ± 8.34 mg ($P = 0.17$, $P = 0.23$ and $P = 0.59$; vs. control groups: 265.02 ± 7.51, 260.82 ± 7.33 and 266.49 ± 8.50), which reached to 294.68 ± 11.23 mg, 291.37 ± 9.04 mg and 298.54 ± 12.40 mg at the end of study ($P = 0.26$, $P = 0.23$ and $P = 0.19$; vs. control groups: 288.94 ± 10.73 mg, 290.58 ± 11.71 mg and 293.26 ± 6.40 mg).

The effects of repeated pre-exposure to DHA+EPA on renal functional parameters (SCr, BUN, CCr and FENa)
As shown in Figure 1, ischemia (45 min) and reperfusion (6, 24 and 48 h) significantly increased serum creatinine (1.84 ± 0.11, 1.67 ± 0.14 and 1.53 ± 0.12 mg/dL, respectively; $P < 0.01$) and BUN (31.54 ± 3.15, 28.71 ± 2.6 and 26.18 ± 3.04 mg/dL, respectively; $P < 0.01$) in comparison with Sham-operated groups with the same time post-operation period (SCr: 0.52 ± 0.07, 0.55 ± 0.04 and 0.50 ± 0.04, respectively, and BUN: 11.36 ± 1.14, 12.08 ± 1.09 and 10.61 ± 0.9, respectively). Two-week pretreatment by oral supplementation with 200 mg/kg/day DHA+EPA decreased serum creatinine (0.84 ± 0.09, 0.78 ± 0.08, and 0.82 ±

![Figure 1](image-url) Renal functional assessment after ischemia reperfusion (IR). (a) serum creatinine (SCr) and (b) BUN was measured after 6, 24 and 48 h of reperfusion or Sham operation in serum samples. (c) creatinine clearance (CCr) was calculated from serum and urinary creatinine concentrations and urinary volume, which was collected during reperfusion period. (d) Fractional excretion of sodium (FENa) was calculated from serum and urinary sodium concentration, urinary volume and CCr. Data are given as Mean ± SEM IR, Docosahexaenoic acid+Eicosapentaenoic acid (gavage; 200 mg/kg/day for 2 weeks before ischemia). **$P < 0.01$ vs. Sham, †$P < 0.05$ vs. IR group.
0.06 mg/dL, respectively; \( P < 0.05 \) vs. IR groups) and BUN (19.72 ± 1.72, 16.62 ± 1.64 and 14.73 ± 1.19 mg/dL, respectively; \( P < 0.05 \) vs. IR groups) after 6, 24 and 48 h of reperfusion.

Creatinine clearance (CCr) is used as an indicator of GFR which significantly decreased after 45-min ischemia and 6, 24 and 48 h of reperfusion (\( P < 0.05 \) vs. Sham-operated groups). DHA+EPA pretreatment (200 mg/kg/day) increased CCr after 6, 24 and 48 h of reperfusion (0.68 ± 0.09, 0.84 ± 0.11 and 0.93 ± 0.12 mL/min, \( P < 0.05 \) vs. IR groups; Figure 1). FENa was calculated to assess proximal tubule function. FENa was increased after IR at the different times of reperfusion in comparison with sham-operated animals (23.0 ± 2.93%, 20.0 ± 2.16% and 22.0 ± 3.04% vs. 5.0 ± 0.76%, 6.0 ± 0.52 and 4.0 ± 0.52%, at 6, 24 and 48 h, respectively; \( P < 0.01 \), Figure 1). DHA+EPA pretreatment significantly decreased FENa after 6, 24 and 48 h of reperfusion (11.0 ± 1.84, 10.0 ± 1.73, and 9.0 ± 1.5%, \( P < 0.05 \) vs. IR groups; Figure 1).

### Renal MDA contents and CAT and SOD activities

Ischemia reperfusion significantly increased renal MDA contents at 6, 24 (\( P < 0.01 \)) and 48 h (\( P < 0.05 \)) after surgery, while pretreatment with DHA+EPA at all mentioned times of reperfusion kept MDA at lower levels (27.81 ± 1.99, 24.96 ± 2.38 and 20.7 ± 2.42 nmol/mg, \( P < 0.05 \) vs. IR groups; Figure 2).

After 6 and 24 h of reperfusion period in the IR groups, SOD activity of renal tissue was significantly decreased (22.02 ± 3.59 and 27.37 ± 3.41 U/mg, \( P < 0.01 \) in comparison with the sham-operated animals). SOD activity remained at a low level after 48 h of reperfusion in IR group (\( P < 0.05 \) vs. sham-operated group); however, it was more than the value of 6 h in IR group (\( P < 0.05 \), Figure 2). DHA+EPA supplementation (200 mg/kg/day) increased SOD activity after 6–48 h of reperfusion (\( P < 0.05 \) vs. IR groups, Figure 2).

Catalase activity was decreased in IR groups after 6–48 h of reperfusion (\( P < 0.05 \) vs. sham-operated groups). The minimum value of CAT activity in IR groups recorded in rats who had experienced 6 h of reperfusion (\( P < 0.05 \) vs. IR group with 48 h of reperfusion period Figure 2). Although CAT activity in DHA+EPA-treated groups was lower than the CAT activity in sham-operated groups during 6–24 h of reperfusion (\( P < 0.05 \) vs. same), but it was more than the activity of CAT in IR groups which experienced the same time of reperfusion (\( P < 0.05 \) vs. IR). After 48 h of reperfusion, CAT’s activity returned to physiological levels and was...
significantly more elevated than the 6 h-reperfusion value in DHA+EPA-pretreated rats ($P < 0.05$, Figure 2).

**Bax and Bcl-2 expression**

The expression of pro-apoptotic protein Bax (21KD) significantly increased after 24 and 48 h of reperfusion in IR groups ($P < 0.05$ in comparison with the sham-operated group; Figure 3), while 6 h-reperfusion did not show any significant change in the expression of Bax protein in IR group. In DHA+EPA-pretreated rats, Bax expression was not changed significantly after 24 and 48 h of reperfusion ($P < 0.05$ vs. IR groups, Figure 3).

Bcl-2 protein expression was not changed after 6–48 h in IR groups. However, pretreatment with DHA+EPA (200 mg/kg/day) increased Bcl-2 expression 24 and 48 h after ischemia ($P < 0.05$ vs. IR groups; Figure 3).

In IR group, Bax expression at 24 and 48 h of reperfusion was significantly more than the 6 h-reperfusion IR group ($P < 0.05$).

In DHA+EPA-treated rats, Bcl-2 expression at 24 and 48 h was significantly more than the Bcl-2 expression at 6 h-reperfusion DHA+EPA group ($P < 0.05$).

**Light microscopy evaluation of renal histological**

Results of histological studies are summarized in Table 1 and Figures 4–6. Grade-0 histological score was observed in 91.1, 95.6 and 88.9% of slides which were prepared at 6, 24 and 48 h after sham operation (Figures 4a, 5a and 6a; Table I). After 6 h of reperfusion in the IR group mild (Grade-1) and moderate (Grade-2) signs of renal tissue injury were observed in 30.0 and 37.8% of slides, which included tubular dilation and interstitial edema in small areas of studied fields ($P < 0.05$ vs. Sham; Table I; Figure 4b). Pretreatment with DHA+EPA for 2 weeks before ischemia had no effect on IR-induced tissue injury after 6 h of reperfusion ($P < 0.05$ vs. Sham; Figure 4c, Table I).

After 24 and 48 h of reperfusion in the IR groups, moderate and severe (Grade-2 plus Grade-3) renal tissue injuries were observed in 67.6 and 63.3% of slices with brush border loss in the major parts of proximal tubules, large number of cell debris and detached epithelial structures, obstructed and swollen tubules ($P < 0.01$ vs. sham-operated groups; Figures 5b and 6b; Table I). DHA+EPA (200 mg/kg/day) significantly reduced tubular damage ($P < 0.05$ vs. IR group; Table 1).
Figure 4 Renal histology 6 h after ischemia or sham operation. Left renal tissues slices were selected from (a) Sham, (b) ischemia reperfusion (IR) and (c) Docosahexaenoic acid+Eicosapentaenoic acid (DHA+EPA) 200 mg/kg/day+IR groups. (a) Sham-operated group showed normal appearance in most of the slices and marked as grade 0 or 1 in pathological scoring system. (b) IR induced mild-to-moderate histological damage in most slices which is shown by cell debris and tubular swelling (black arrows). (c) DHA+EPA decreased IR-induced renal injury (mild) with rare cellular debris in tubular spaces (black arrows). (H & E staining, original magnification × 100).

Figure 5 Renal histology 24 h after ischemia or sham operation. (a) Sham-operated group showed normal appearance in most of the slices (H & E staining, original magnification × 40). (b) Ischemia reperfusion (IR) induced moderate-to-severe histological damage in most slices; disappeared brush border in main parts of proximal tubules, large number of cell debris and detached epithelial structures which obstructed tubules and tubular swelling (black arrows) were the dominant feature of slices prepared 24 h after ischemia. (c) Docosahexaenoic acid+Eicosapentaenoic acid decreased IR-induced renal injury (mild to moderate) with diffused cell debris and tubular swelling (black arrows). (H & E staining, original magnification × 100).

Figure 6 Renal histology 48 h after ischemia or Sham operation. (a) Sham-operated group showed normal appearance in most of the slices. (b) Moderate-to-severe histological damage remained after 48 h of reperfusion in renal tissue; damage to brush border, epithelial cell debris and dilated tubules were observed in major parts of proximal tubules (black arrows). (c) Docosahexaenoic acid+Eicosapentaenoic acid decreased ischemia reperfusion-induced renal injury (mild) with sparse cell debris and tubular swelling (black arrows). (H & E staining, original magnification × 100).
and Figures 5c and 6c) and restricted cellular debris and tubular swelling to some parts of studied fields.

**DISCUSSION**

To determine, for the first time, the role of DHA+EPA supplementation on renal IR injury, the present study was designed to evaluate renal function and histology along with oxidative stress and protein expression of the pro- and anti-apoptotic Bax and Bcl-2 proteins from 6 to 48 h after ischemia. The results showed that 2-week pretreatment with DHA+EPA improved renal function and decreased oxidative stress started from the first hours after ischemia and lasted for the duration of the study (48 h). This effect was accompanied by significant modulation of apoptotic protein expressions and attenuation of histological damage after 24 h of reperfusion. Limited apoptosis and histological damage seems to be a protracted response to decreased oxidative stress at the first hours of reperfusion in the DHA+EPA-treated rats.

Superoxide dismutase and CAT enzymes cooperate to protect against ROS and oxidative stress by, respectively, converting the superoxide anion into hydrogen peroxide and by converting H2O2 into water. Any imbalance between ROS production and antioxidant defences will lead to oxidation of membrane lipids, proteins and DNA [24]. Increased renal MDA contents after ischemia confirmed lipid peroxidation owing to increased oxidative stress which may be the consequence of the observed decreased SOD and CAT activities after IR (Figure 2). Increase in renal oxidative stress and loss in SOD and CAT activities were associated with decreased CCR and increased Scr, BUN and FEna (Figures 1 and 2). This is supported by a study by Yamanobe et al. [25] showing that susceptibility to ARF was increased after 45 min of renal ischemia in SOD1-deficient mice (SOD−/−).

In DHA+EPA-treated rats, levels of SOD and CAT activities remained higher during the 48-h reperfusion period which was associated with decreased MDA levels and improved renal function (Figures 1 and 2). Studies by Kim et al. showed that both SOD mimetic and irradiation therapies reduced plasma creatinine, lipid peroxidation and tissue hydrogen peroxide by increased SOD activity after renal ischemia [26,27].

Renal tubular cell apoptosis is well documented in experimental models of acute ischemic and toxic injury [28,29] and in tubules of transplanted kidneys in humans [30]. In recent studies, administration of compounds possessing SOD and CAT activities to endothe-

and human lymphoma U937 and HH cells in vitro attenuated radiation- and oxidative stress-induced apoptosis [31,32]. In the present study, prevention of Bax (pro-apoptotic protein) overexpression after 24 and 48 h of reperfusion, in the DHA+EPA-pretreated groups, concomitantly with the increases in SOD and CAT activities suggest that these enzymes may reduce the oxidative stress-induced apoptosis. It has been shown that consistent production of H2O2 decreased gastric epithelial cells’ viability and increased DNA fragmentation while Omega-3 fatty acids prevented oxidative stress-induced cell death, DNA fragmentation and expression of Bax [33].

During hypoxia pro-apoptotic proteins, Bax with a dominant role, translocate from the cytoplasm to the mitochondrial membrane and permeabilize the outer mitochondrial membrane, leading to cytochrome c (Cyt c) release which triggers apoptosis downstream cascade [34,35]. Conversely, anti-apoptotic protein Bcl-2 prevents mitochondrial membrane rupture as a result of mitochondrial matrix swelling [36]. In the present study, we observed increased Bcl-2 protein expression in the DHA+EPA-treated group after 24 and 48 h of reperfusion (Figure 3), concomitantly with DHA+EPA decreased histological damage (Figures 5 and 6) after 24 and 48 h of reperfusion. Therefore, this indicates that DHA+EPA-mediated up-regulation of Bcl-2 expression could play a more important role in the protection of the kidney at the latter phases of reperfusion (Figures 3, 5 and 6). In Support of these findings, Burns et al. [37] suggested that in specimens from cadaveric donor transplants apoptosis occurred more frequently in post-reperfusion compared with pre-reperfusion samples.

In conclusion, the results of the present study showed that DHA+EPA pretreatment for 2 weeks increases renal tissue tolerance to IR injury and prevents tubular cell apoptosis up to 48 h after ischemia. Because the severity of the tubular epithelial cell apoptosis predicts early graft function [38], and induction of anti-apoptotic protein expression inhibits proximal and distal tubular apoptosis and predicts renal function [8,9], pre-ischemic treatments with molecules, such as DHA+EPA, reducing Bax overexpression or decreasing the Bax/Bcl-2 ratio would have important clinical implications [12,39].

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CONFLICT OF INTEREST

Shahid Beheshti University of Medical Sciences founded all stages of present work, and there were no conflicts of interest to any other Institution.

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