Neuroprotective Effects of Postconditioning on Lipid Peroxidation and Apoptosis after Focal Cerebral Ischemia/Reperfusion Injury in Rats

ABSTRACT

AIM: Apoptosis after cerebral ischemia/reperfusion (I/R) injury leads to the process of cell death. The ideal therapeutic approach would target the apoptosis after I/R. Ischemic postconditioning is a recently discovered neuroprotective strategy that involves the application of brief mechanical reperfusion with a specific algorithm at the onset of reperfusion following an ischemic period.

MATERIAL and METHODS: Transient MCAo was performed on male SD (275±25g) rats with intraluminal thread insertion for 2hrs. Rats (n:36) were treated with postconditioning after 60 minutes of occlusion. The postconditioning algorithm was 30 secs of brief reperfusion followed by 30 secs of MCAo and this cycle was repeated 3 times at the onset of reperfusion.

RESULTS: After I/R injury, % change of the malonyldialdehyde (MDA) levels in the cortex, which is an index of lipid peroxidation, was found significantly higher in the I/R group. On the other hand postconditioning upregulated Bcl-2 and Bax translocation to the mitochondria, and caspase-3 activity and also reduced oxidative stress levels.

CONCLUSION: These findings indicated this neuroprotective effect is most likely achieved by antiapoptotic mechanisms through caspase pathways.

KEYWORDS: Antioxidant enzymes, Ischemia reperfusion injury, Lipid peroxidation, Neuroprotection, Postconditioning

ÖZ

AMAÇ: Geçici fokal serebral iskemi modeli uygulanan sıçanlarda ardkoşullama uygulamasının lipid peroksidasyonu ve apoptozis üzerine etkisinin araştırılması.

YÖNTEM ve GEREC: Geçici fokal serebral iskemi, 32 adet (275±25g) Sprague-Dawley erkek sıçanlarda intraluminal filament yöntemi ile orta serebral arterde geçici okluzyon (OSAo) oluşturuldu. OSAo doğrulanması ipsilateral parietal kortekte Laser Doppler Flowmetre monitörizasyonu ve takiben nörolojik muayene ile yapıldı. Deney grupları; sham; iskemi ve ardkoşullama grubu olarak planlandı. 120 dakikalık okluzyon takiben 24 saat reperfüze edilerek dekapite edildi. İmmünohistokimyasal analiz için bcl-2, kaspaz 3, kaspaz 9 ve bax immün boyamanı yapıldı. Lipid peroksidasyonu saptamak içinde her iki hemisferde dokuda malonyldialdehit (MDA) seviyeleri saptandı.
INTRODUCTION

Apoptosis after cerebral I/R injury is one of the major pathways that lead to the process of cell death (21). In response to the oxidative load in mitochondria, the outer membrane of mitochondria becomes permeabilized (15) resulting in the translocation of Bax from the cytosol to the mitochondria and the release of cytochrome c, normally confined to the mitochondrial intermembrane space (16,20). This proapoptotic protein translocation is controlled by the family of Bcl-2 proteins (17). Release of cytochrome c into the cytosol leads to the formation of the apoptosome, a complex composed of apoptotic-protease activating factor-1, procaspase-9, and ATP (18). The apoptosome permits the autoactivation of procaspase-9, which is followed by the activation of procaspase-3 (9). Active caspase-3 leads to DNA fragmentation (18). Some studies have demonstrated that apoptosis contributes to the development of ischemic infarction with DNA fragmentation (19). Thus, the ideal preventive or therapeutic approach should target apoptosis after cerebral I/R injury.

Revascularizing the occluded vessels allowing timely reperfusion is one of the strategies currently being pursued for acute stroke. However, reperfusion itself generates an overproduction of reactive oxygen species or free radicals, leading to reperfusion injury which is a complex process involving endothelial and microvascular dysfunction, impaired blood flow, metabolic dysfunction, cellular necrosis and apoptosis (36). Despite extensive research on reperfusion injury treatment in the past several decades, only a few neuroprotectants have been successfully transferred from basic research into clinical application; hypothermia (4) and preconditioning (8,22). Recently, Zhao et al., described an another novel way for treating ischemic infarct in experimental focal ischemia models in rats; ischemic postconditioning (35).

Ischemic postconditioning is a neuroprotective strategy that involves the application of brief mechanical reperfusion with a specific algorithm at the onset of reperfusion following an ischemic period. Alternating periods of reperfusion-reocclusion at the beginning of reperfusion is defined as postconditioning and provides a new insight into molecular mechanisms responsible for endogenous neuronal protection and this indicates a necessity for new strategies to increase the durability of brain cells to ischemic insult (8,14,27,36).

Similar pathways and molecules take place in pre- and postconditionings but their roles and timing are different in each conditioning. Understanding the neuroprotective effects of mechanisms underlying conditionings has been elusive, but NMDA receptor activation, nitric oxide, inflammatory cytokines, and suppression of the innate immune system appear to have a role. Reactive oxygen species and classical ligand stimuli play a role in postconditioning with KATP channels and protein kinase C pathways acting as mediators.

In this study, we aimed to investigate the potential neuroprotective effects of ischemic postconditioning on the lipid peroxidation and apoptosis using a transient focal cerebral I/R injury model in rats.

MATERIALS and METHODS

All procedures were performed in the Neurovascular Research Laboratory of the Department of Physiology and Neurosurgery. Animal care was conducted with the prior approval of the Animal Experimental Ethics Committee of Uludag University (31.01.2006/1).
Transient focal cerebral ischemia model:

All the animals were housed in individual cages at a constant temperature under a controlled 12-hour light/dark cycle with free access to water and food. This study used 36 adult male Sprague-Dawley rats weighing 275±25g. Anesthesia was induced with 4% isoflurane, and then maintained with 2% isoflurane in a mixture of 70% N2O and 30%O2, with spontaneous breathing. Rectal temperature was maintained 37°C during and after four hours of surgery until they completely recovered from the effects of anaesthesia at 37°C on a feedback-regulated heating pad (CMA Probe, Sweden). All animals were subjected to 2 h transient focal cerebral ischemia followed by 24 h reperfusion, using an intraluminal suture technique described by Kawamura et al. (11). In brief, the left common carotid artery, internal carotid artery, and external carotid artery were exposed surgically. A 4-0 monofilament nylon suture (Ethilon, Ethicon Inc., Somersville, NJ, USA) with a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. After 120 minutes of MCAo, the suture was removed to restore blood flow (occlusion and reperfusion confirmed by changes of local cerebral blood flow). Local cerebral blood flow (LCBF) was monitored by laser-Doppler flowmeter (Vasamedic, Blood Perfusion Monitor, Model BPM 433-1, St Paul MN, USA) with the use of a flexible probe over the skull. The probe used was 0.8mm diameter and placed in a 1mm burr-hole made at the left temporal bone. LCBF was measured before ischemia, during MCAo, and during reperfusion. The experiment used only rats whose LCBF values were reduced more than 50% after insertion of the thread and remained stable at that level after throughout the occusion period. Core body temperatures were monitored with a rectal probe and maintained at 37°C during the whole procedure. All surgical procedures were performed under an operating microscope (Opmi 99, Carl Zeiss, Germany).

Oclusion was also tested with post-procedural neurological examination according to Bederson et al. (1). Neurological scores were evaluated by a neuroscientist who was blinded to the groups. Only rats scoring less than 6 were used for the subsequent experiments (1).

Randomly selected animals were divided into 3 groups as follows: Group 1 (n: 4) Sham; Group 2 (n: 16), I/R with 120 minutes of MCAo followed by 24 hrs of reperfusion, and Group 3 (n: 16), I/R plus postconditioning group. Postconditioning was performed after 120 minutes of MCAo, at the beginning of reperfusion. For the postconditioning study, reperfusion was established for 30 seconds, after which the MCA was occluded again for 30 seconds, followed by another 3 cycles of the same period of reperfusion and occlusion as suggested by Zhao et al. (36). The rats were allowed to recover 2h after MCAo and postconditioning. Rats were sacrificed at 24hrs after reperfusion.

Assessment of neurological symptoms

All animals were assessed according to Bederson’s 4-score scale 1 hour after surgery; Grade 0: No observable deficit, Grade 1: Forelimb flexion, Grade 2: Decreased resistance to lateral push (and forelimb flexion) without circling, Grade 3: Same behavior as grade 2, with circling. Only rats that scored less than 6 points were used for the subsequent experiments. The rats were sacrificed at the end of a 24-hour reperfusion in all groups. Furthermore, the animal was also excluded from the study when there was evidence of subarachnoid hemorrhage on extraction of brain tissue.

Morphological studies and immunohistochemistry

Tissue: Brain tissues were obtained from rats (n: 36) following perfusion fixation with 4% paraformaldehyde in tris buffer (pH 7.4). Brains were quickly removed and postfixed in the same fixative overnight at 4°C. Fixed brains were dehydrated in a graded series of alcohols, cleared in xylene, impregnated with paraffin and embedded in paraffin blocks. Sections five micrometers thick were taken throughout the rostro-caudal extent of the hypothalamus.

Antigen Retrieval (AR): Preliminary immunohistochemical tests to determine effective working dilutions of caspase-3, caspase-9, Bax and Bcl-2 antibodies (without using AR) resulted in very weak to none immunoreactivity; thus AR was needed to achieve the optimal immunostaining. Sections were mounted on poly-L-lysine-coated slides and were deparaffinized with xylol, passed through graded alcohols, and rinsed successively in distilled water. This treatment was followed by the use of 1% hydrogen peroxide in order to block endogenous
peroxidase. The sections were then washed and processed using the AR procedure, which was carried out in a microwave oven.

**Caspase 3, Caspase 9, Bax and Bcl-2 Immunohistochemistry:** Following the blocking incubation in normal horse serum (10% in Tris-HCl buffer containing 0.1% Na-azide and 0.2% Triton X-100), sections were incubated overnight at 21°C with primary antibody (rabbit anti-caspase-3, caspase-9, Bax or Bcl-2 (Santa Cruz Biotechnology, CA)). Antibodies were diluted in the above blocking buffer at a dilution of 1:100, 1.100, 1:200 and 1:50, respectively. Incubation was terminated by three washes in Tris-HCl buffer. Sections were then incubated for 60 min. in the affinity purified and cross-absorbed biotin-conjugated donkey anti-rabbit serum (1:250, Jackson Immunoresearch Labs, PA). Sections were processed for bright field immunohistochemistry using the Elite ABC Kit (Vector Labs, Burlingame, CA). Diaminobenzidine (50 mg) with 5 μl H2O2 in Tris-HCl buffer (100 ml) was used as cromogen to visualize the antigen-antibody complex. After several washes in Tris-HCl buffer, sections were dehydrated in graded alcohol, cleared in xylol, mounted and visualized with an Olympus BX-50 photomicroscope. Control experiments included omission of primary antibody and substitution of the primary antibody by normal serum, as well as extensive tests of cross-reactivity of the secondary antibodies. Positively stained cells were counted in up to ten different areas per animal and the frequency of positive cells was determined as a percentage of total cells. Both the intensity of staining and the distribution of positively stained cells were taken into account in scoring. Protein expression was therefore evaluated by a semi-quantitative scoring system. The intensity and distribution of expression were evaluated as follows: Grade 0: no expression, Grade 1: weak expression, 0-25% (+); Grade 2: moderate expression, 26-50% (++; Grade 3: intense expression, 51-75% (+++); Grade 4: more intense expression, 76-100% (++++).

**Determination of Oxidative Stress (Malondialdehyde activity)**

Brain tissue MDA levels were determined by the thiobarbituric acid method, and expressed as nmol MDA/mg protein. Brain tissue homogenates were prepared as follows; 0.25 g of tissue sample was homogenized in 2.5 ml of ice-cold 1.15% potassium chloride buffer. The amount of lipid peroxides was measured as the production of MDA. Aliquots of homogenates were then used for analysis of lipid peroxides (5).

The experimental procedures for each group are shown in Figure 1.

**Statistical analysis**

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The nonparametric Kruskal-Wallis test was used for comparison of the degree of immunohistochemical staining. The nonparametric Mann-Whitney U test was used to define the differences between the groups. A value of p<0.05 was considered as significant.

**RESULTS**

There were no differences in the body weight of the rats in any of the groups (Group 1 265.50±7.76g; Group 2 277.41±14.14g; Group 3 279.47±13.43g; p>0.05) and body temperatures during the experiment (Group 1 36.95±0.12°C; Group 2 36.95±0.15°C; Group 3 36.93±0.11°C; p>0.05). Immediately after MCAo, LCBF measurements showed sharp drops of over 50% as compared to control (Group 2 73.38%; Group 3 78.70%; p<0.05). All groups exhibited significant decreases in rCBF values after MCAo. The pre-MCAo values for Group 2 were 80.58±33.57 ml LD/100g/min and Group 3 74.25±34.80 ml LD/100g/min (p>0.05). After MCAo values were 15.82±4.19 ml LD/100g/min for Group 2 and 13.40±4.04 ml LD/100g/min for Group 3. There was no significant change in LCBF between I/R and postconditioning groups after occlusion (p>0.05).

There were no significant differences in neurological scores between the groups at one hour.
after MCAo (Group 2: 2.85±0.25; Group 3: 3.00±0.01; p>0.05). Sham operated rats did not have any deficits.

The MDA level in the right hemisphere, an index of lipid peroxidation, was significantly higher in the I/R group. There was a significant reduction % change in MDA levels in the postconditioning group compared with the IR group (Table I).

There were significant differences for immunohistochemical scorings between the groups for caspase 3, caspase 9 and bax, Bcl-2. Caspase-3 and caspase-9 activities were noticeably increased but not Bax levels in the I/R group compared to sham. Antiapoptotic Bcl-2 protein and Bax levels in the mitochondria were significantly increased in the postconditioning group compared to I/R group (p<0.01; p<0.05 respectively) but no differences were observed for caspase 3 and 9 (Table II).

Table I: There were significant differences of hemispheric malonyldialdehyde (nmol MDA/g) and % change levels between the groups (p<0.05). Although not statistically significant, postconditioning reduced % change MDA levels 39.61% compared to I/R group.

<table>
<thead>
<tr>
<th>MDA level (nmol/g)</th>
<th>Right Hemisphere</th>
<th>Left Hemisphere</th>
<th>Change %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>297.00±53.39</td>
<td>307.25±62.87</td>
<td>3.30</td>
</tr>
<tr>
<td>Group 2</td>
<td>279.75±50.03</td>
<td>203.75±42.56</td>
<td>27.29*</td>
</tr>
<tr>
<td>Group 3</td>
<td>307.50±41.21</td>
<td>272.25±39.15</td>
<td>10.81*</td>
</tr>
</tbody>
</table>

Table II: Bax, Bcl-2, Caspase 3, Caspase 9 expressions of the groups after cerebral ischemia/reperfusion (I/R) injury. Postconditioning treatment significantly upregulated antiapoptotic Bcl-2 (p<0.01).

<table>
<thead>
<tr>
<th></th>
<th>Bcl-2</th>
<th>Bax</th>
<th>Caspase3</th>
<th>Caspase9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.25±0.50</td>
<td>0.50±0.57</td>
<td>0.25±0.50</td>
<td>0.25±0.50</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.50±0.53</td>
<td>0.50±0.53</td>
<td>1.37±0.51</td>
<td>2.00±0.00</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.50±0.53*</td>
<td>1.12±0.35</td>
<td>1.60±0.74</td>
<td>1.87±0.64</td>
</tr>
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</table>

**DISCUSSION**

The current lack of clinical treatment for acute stroke necessitates the exploration of novel concepts that may eventually lead to clinical application. One of these recent concept is ischemic postconditioning (36,38) that refers to an interference of a series of brief, repetitive occlusion and release of the cerebral blood vessels after reperfusion. It has been demonstrated that rapid ischemic postconditioning performed immediately after reperfusion reduces infarct in focal cerebral ischemia (6,7,29,36) and this has been confirmed by a number of other groups in global (24,32) and focal ischemia (23,34). The brain relies upon internal defense mechanisms for protection from injurious stimuli. Postconditioning, defined as brief periods of reperfusion alternating with re-occlusion applied during the very early minutes of reperfusion, mechanically alters the hydrodynamics of early reperfusion. Reperfusion injury is a complex process involving endothelial and microvascular dysfunction, impaired blood flow, metabolic dysfunction, cellular necrosis and apoptosis (29,33,36). While cerebral ischemic preconditioning has been known to protect against strokes for some time, early and delayed postconditioning has recently been shown to reduce ischemic damage (25,33,36). Zhao et al. were the first to document that ischemic postconditioning reduced infarct size in cerebral I/R injury (36).

Gao et al. recently demonstrated that the protective effects of postconditioning are determined by the number of cycles of reperfusion/occlusion, the duration of reperfusion/occlusion, and the onset time of postconditioning. Its protection, though similar to that of rapid preconditioning, is less than that of delayed preconditioning (6).

Our previous study has established that postconditioning reduces infarct size after focal ischemia (29) but the way postconditioning effects the antiapoptotic mechanisms was not addressed. Xing et al. supported the speculation that postconditioning provides neuronal protection by inhibiting apoptosis that has been proven to activate caspase-3. Our study and also Xing et al. clearly showed that postconditioning increased the level of antiapoptotic Bcl-2 protein in the mitochondria, probably inhibited Bax translocation to the mitochondria, and inhibited cytochrome c release from the mitochondria to the cytosol (33). It was presumed that the aforementioned mechanism led to a decrease in the activation of caspase (33). There is increasing evidence that some neuronal death after brain ischemia is mediated by the action of cysteine-requiring aspartate directed proteases (caspases), the proteases responsible for apoptosis in mammals. Caspase-mediated neuronal...
death is more extensive after transient rather than permanent focal brain ischemia and may contribute to delayed loss of neurons from the penumbral region of infarcts. The intracellular processes that contribute to caspase-mediated neuronal death following ischemia all have the potential to be treated by logical therapy. It is known that cytochrome c is released from the mitochondria to the cytosol and plays a key role in the initiation of apoptosis through the activation of the caspase cascade (18). By regulating the Bcl-2 (antiapoptosis)/Bax (proapoptosis) balance, the Bcl-2 family maintains mitochondrial stabilization (10). Our results and others suggested that the mitochondrial pathway was an important target for postconditioning (33). The anti-apoptotic protein Bcl-2 improves neuron survival following various insults, and is protective even when administered after stroke in a rat model of focal ischemia (37). The precise molecular mechanisms underlying protection by the Bcl-2 family are not yet completely understood.

The early moments of reperfusion are important in the pathogenesis of posts ischemic injury. Previous studies have demonstrated that ischemic postconditioning reduces cerebral I/R injury (36, 33). It has been suggested that similar pathways and molecules take place in pre and postconditioning but their roles and timing are different in each condition (36). Reperfusion has the potential to introduce additional injury that is not evident at the end of ischemia per se, i.e. reperfusion injury. Reperfusion injury is expressed as endothelial and microvascular dysfunction, impaired blood flow, metabolic dysfunction, cellular necrosis, and apoptosis. There is an impressive array of mechanisms contributing to reperfusion injury. Postconditioning also stimulates endogenous mechanisms that attenuate the multiple manifestations of reperfusion injury. These mechanisms include ligands, such as adenosine and opioids that act as proximal triggers to stimulate molecular pathways involving mediators such as protein kinase C, mitochondrial ATP-sensitive potassium channels, and survival kinases (35). Postconditioning has been shown to inhibit the mitochondrial permeability transition pore. Postconditioning may also inhibit deleterious pathways such as p38 and JNK mitogen-activated protein (MAP) kinases and attenuate the damage to endothelial cells and cardiomyocytes from oxidants, cytokines, proteases, and inflammatory cells.

Clinical studies in patients with acute myocardial infarction have demonstrated that postconditioning was effective in reducing infarct size (31). Postconditioning indirectly supports the concept of reperfusion injury in animal models of ischemia-reperfusion and in patients, and exerts cardio protection that is equivalent to that of ischemic preconditioning.

Apoptosis after cerebral I/R is one of the major pathways that lead to the process of cell death. The outer membrane of mitochondria becomes permeabilized resulting in the translocation of Bax from the cytosol to the mitochondria and the release of cytochrome c, normally by the family of Bcl-2 proteins. (17) Release of cytochrome c into the cytosol leads to the formation of the apoptosome, a complex composed of apoptotic-protease activating factor-1, procaspase-9, and ATP (18). The apoptosome permits the auto activation of procaspase-9, which is followed by the activation of procaspase-3. Active caspase-3 leads to DNA fragmentation (18). Some studies have demonstrated that apoptosis contributes to the development of ischemic infarction with DNA fragmentation. (19). Thus, the ideal preventive or therapeutic approach would indeed target apoptosis after I/R. Ischemic postconditioning is defined as a series of rapid intermittent interruptions of blood flow in the early phase of reperfusion that mechanically alters the hydrodynamics of reperfusion. Developments in cardiac physiology have indicated that postconditioning significantly reduces infarct size and inhibits inflammation and apoptosis (2,3,12,13,28,30,38). In addition, a recent clinical report demonstrated that postconditioning after coronary angioplasty and stenting protected the human heart during acute myocardial infarction (26).

In our previous study for investigating the neuroprotective effects of pre-and postconditioning applied alone and together, we observed for the 3x30 secs postconditioning process applied 2 hours after MCA occlusion that the 3x30 secs postconditionings were consecutive so that each 30 sec occlusion in fact served as the preconditioning of the following 30 sec occlusion (29). In this way, the protective effect stimulated during the previous preconditioning period was strengthened in the following period as suggested by Zhao et al. (36). This effect was also reflected in the infarct volumes as we observed in our previous study (29). There was a significant decrease
in infarct volumes in the both pre and postconditioning groups alone and together.

As we noticed in this and in our previous studies, preconditioning, postconditioning and pre/postconditioning have preventive effects on cerebral I/R lesions (29). Ischemic pre/postconditioning provides a new insight into molecular mechanisms responsible for endogenous neuronal protection and this indicates a necessity for new strategies to increase the durability of brain cells to ischemic insult. Although it has never been named before, multiple short periods of temporary clip applications during early aneurysm surgery is a kind of postconditioning and may protect from subsequent development of ischemia. Cerebral ischemic postconditioning may protect against stroke, but is clinically feasible only when the occurrence of stroke is predictable. The prediction of possibility of development of stroke in neurosurgical practice is as commonly seen during early aneurysm surgery and producing of brief, repetitive intermittent clip occlusions and release of the main artery after reperfusion may prevent from subsequent vasospasm/ischemic injury after surgery. Producing brief postconditioning during endovascular coil interventions for early treatment of ruptured intracranial aneurysms or stenting carotid artery occlusions may also similarly provide neural protection from ischemia as reported during coronary angioplasty and stenting procedures (26). Both preconditioning and postconditioning may be applicable to certain clinical settings for patient subjected to surgery and endovascular therapy associated with carotid artery or other cerebral vessel occlusion and revascularisation. it is essential to know which neuroprotectant, preconditioning or postconditioning provides better protection or whether a combination of both or with combination of drugs provide synergistic effects to decide on the clinical applications of these protective strategies.

We demonstrated that ischemic postconditioning provides neuroprotection after focal stroke probably by reducing apoptosis and free radical products. In our study, we tested only 6 cycles of 30 seconds of reperfusion followed by 30 seconds of ischemia. An interval of 30 seconds was referred to in the Zhao et al. study (36). Whether postconditioning played its role in an “on-off” or a “dose dependent” manner was not fully elucidated in studies, and 30 seconds may not afford the maximal protective effect against cerebral I/R injury if postconditioning acts in a “dose-dependent” style. Thus, the exact number of optimal intervals and cycles and timing may also need additional investigation. Ren et al. (25) showed that delayed postconditioning performed at 3h and 6h after stroke robustly reduced infarct size, with the strongest protection achieved by delayed postconditioning with 6 cycles of 15 min occlusion/15 min release of the ipsilateral common carotid artery occlusion executed from 6h. The authors found that the delayed postconditioning provided long term protection for up to 2 months by reducing infarction and improving outcomes of the behavioral tests, improving metabolism, and reduced edema and blood brain barrier leakage.

In conclusion, our study demonstrated that postconditioning attenuated apoptosis in focal cerebral I/R injury. This neuroprotective effect was associated with inhibiting apoptosis molecules of the mitochondrial pathway and activating endogenous protective molecules.

REFERENCES


