Cellular neuroprotective mechanisms in cerebral ischemia: related mitochondrial functions and protection offered with Selenium.
INTRODUCTION

Mode of neuronal death caused by cerebral ischemia and reperfusion appears on the continuum between the poles of catastrophic necrosis and apoptosis. Ischemic neurons exhibit many biochemical hallmarks of apoptosis but remain cytologically necrotic. The position on this continuum may be modulated by the severity of ischemic insult. The ischemia-induced neuronal death is an active process (energy dependent) and is the result of activation of cascades of detrimental biochemical events. These events include perturbation of calcium homeostasis leading to increased excitotoxicity, malfunction of mitochondria, elevation of oxidative stress leading to DNA damage, alteration in proapoptotic gene expression and activation of effector cysteine proteases (caspases) and endonucleases leading to final degradation of the genome. All these contribute to the functional deterioration of outputs that is distinguished by behavioral deficits.

During cerebral ischemic cascade, a unifying factor, which leads to mitochondrial dysfunction is lack of oxygen followed by decrease in ATP production that leads to mitochondrial dysfunctions. Inhibition of mitochondrial respiration triggers the generation of lactate and hydrogen ions (H\(^+\)), leading to deregulation of cellular ion metabolism with intracellular calcium accumulation which is a hallmark of neuronal damage induced by ischemia/reperfusion (IR) (Choi 1995, Fiskum 2000, Silver and Erecinska 1992). Mitochondria are compartments of numerous metabolic processes, may be viewed as “Ca\(^{2+}\) buffers”, respiring polarized mitochondria rapidly accumulate Ca\(^{2+}\) from the cytosol (Gunter and Pfeiffer 1990). Excessive mitochondrial Ca\(^{2+}\) accumulation leads to increase in reactive oxygen species (ROS) generation (Maciel et al. 2001), damaging the function of the electron transport chain. The Ca\(^{2+}\) overload leads to the opening of the mitochondrial permeability transition pore (MPTP) in its high conductance state and the release of apoptosis-inducing factors, such as cytochrome c (Brustovetsky et al. 2002), inducing cell destruction. Cytochrome c release from mitochondria and the subsequent activation of caspases 9 and 3 are critical steps (Plesnila et al. 2004) in cerebral ischemic damage. Thus the ischemic brain is subjected to multiple factors that result in damage to its cellular constituents and the neural networks that form the bases of behavioral deficits.

During cell stress various stress proteins are induced in the cell to combat the stressful conditions, amongst one of them is the heat shock protein 70 (Hsp 70). They have a role in cytoprotection against various types of stresses as ischemia (Kelly and Yenari 2002). Their induction in the brain plays a critical role in ameliorating tissue damage (Nowak and Jacwicz 1994, States et al. 1996, Yasuda et al. 2005). Mitochondrial damage appears to be central in the development of ischemic cell death, very few neuroprotective strategies selectively target mitochondria (Friberg and Wieloch 2002). Thus, mitochondria seem to play an important role in orchestrating cell death mechanisms following cerebral ischemia. However, it is still not clear which are the key mechanisms that cause mitochondrial dysfunction and lead ultimately to cell death. Selenium, was taken for the study because of its myriad effects on various cellular mechanisms. In this study, we employed histopathological studies also to describe changes following IR and changes after selenium supplementation. The purpose of this study was to test the efficacy of selenium on postischemic Ca\(^{2+}\) accumulation, caspase activation, ATP and heat stress proteins expression.

MATERIAL AND METHODS

Drugs and Chemicals
Sodium selenite, Fura-2 and ATP kit was procured from Sigma-Aldrich Chemicals Pvt. Ltd., USA. CasPASE™ Apoptosis assay Kit purchased from Geno Technology, Inc., USA. Hsp 70 antibody [5A5] Mouse Monoclonal IF, IP, WB. ab2787, USA. Other chemicals NaCl, KCl, MgCl₂, CaCl₂, Tris, glucose were of analytical reagent grade.

Animals

Male Wistar rats weighing 300-350 g were obtained from Central Animal House, Jamia Hamdard, New Delhi. They were housed in polypropylene cages in air-conditioned room and allowed free access to pelleted diet and water ad libitum. The animals were used in accordance with the procedure approved by the Animal Ethics Committee of Jamia Hamdard, New Delhi.

Induction of Ischemia

It was done according to the method of Longa et al. (1989) as modified by Salim et al. (2003).

Experimental Protocol

Animals were divided into three groups each having eight animals for a period of 7 days. The first group served as sham and saline was given orally, second was MCAO i.e., ischemia was induced for 2 h followed by reperfusion for 22 h, third group was pretreated for 7 days with sodium selenite (0.1mg/kg, i.p) followed by MCAO for 2 h and reperfusion for 22 h i.e., MCAO+S. After the completion of the reperfusion period, animals were sacrificed and brain parts were dissected out.

Isolation of Brain Mitochondria and Synaptosomes

The brain mitochondria were prepared according to the method described by Nagy et al. (1984). A 10% homogenate (w/v) was prepared in isolation buffer (0.32 M sucrose; 5 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid and 0.1 mM EDTA-K⁺; pH 7.5) with a Teflon-glass homogenizer at approximately 1000 rpm with 5 strokes up and 5 strokes down. The homogenate was centrifuged at 1,000g for 10 min at 4°C and pellet (P₁) was discarded. Supernatant (S₁) was further centrifuged at 4°C for 20 min at 12,000g. Pellet (P₂) were resuspended in isolation buffer (3 ml/g of original wet tissue). The pure mitochondria and synaptosomes were isolated on the percoll gradients of 8.5, 10 and 20% (2.0, 4.0 and 4.0 ml) in buffer II (0.25M sucrose, 5 mM HEPES, and 0.1 mM EDTA-K⁺ pH 7.2). Samples were centrifuged at 15,000g for 20 min at 4°C. Synaptosomes was obtained at the junction of 10%/16% percoll and collected with the help of wide-tip Pasteur pipette and analyzed without further purification. The pure mitochondria were obtained at the bottom layer of percoll (20%), which was resuspended in 10 ml of isolation buffer and further centrifuged at 15,000 g for 10 min at 4°C. The pellet was resuspended in appropriate amount of buffer and used for mitochondrial studies.

BIOCHEMICAL ESTIMATIONS

Adenosine 5-triphosphate (ATP) Quantitation in Mitochondria

ATP content was determined in mitochondria by using ATP Sigma-Aldrich bioluminescent commercial kit.

Determination of [Ca²⁺]i in Synaptosomes

Intracellular calcium [Ca²⁺]i was measured by the method as described by Tretter et al. (1997). The synaptosomes were loaded with fura-2 by incubation in the standard medium (NaCl 140 mM, KCl 3 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, Tris-HCl (pH 7.4) 25 mM, glucose 10 mM) containing 8 μM fura-2 acetoxymethoxy ester at 37 °C (4 mg/ml protein) for 60 min. Synaptosomes were
then washed three times with standard medium and again resuspended in the standard medium to give 8 mg/ml protein concentration. 50 μl aliquots in 2 ml of standard medium were used for the determination. The fluorescence of fura-2 was measured as described by Deri et al. (1993) in a fluorescence spectrometer of 96 well plate reader (Perkin Elmer-LS50B). The standard curve of Ca$^{2+}$, 0.02, 0.1, 1.0 and 3.0 mM CaCl$_2$ was used. The maximum fluorescence ($F_{\text{max}}$) of fura-2 with the 2 μM inomycine and minimum fluorescence ($F_{\text{min}}$) with 100 μM EGTA was used. The binding capacity ($K_d$) of fura-2 with Ca$^{2+}$ was 135 used for the calculation of Ca$^{2+}$ in synaptosomes, as described by Grynkiewicz et al. (1985).

Calculation:

$$\text{Calcium (nmol Ca}^{2+}\text{ synaptosomes) = } K_d \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \right)$$

**Assay of Caspase Activities**

The caspase activities were studied by using CasPASE™ Apoptosis assay Kit. The assay was based on the detection of cleavage of a synthetic substrate, which has 7-amino-4-trifluoromethyl coumarin (AFC) at C-terminal. When liberated from the peptide LETD-AFC produces an optical change which was detected by 96 well plate reader of fluorometer (Perkin Elmer-LS50B) at 360-390 nm excitations and 510-550 nm emission filters.

**Isolation of Heat Stress Proteins (Hsp 70)**

Hsp 70 was isolated from rat brain parts as suggested by Yang and Lin (1999). Briefly, the animals were killed by decapitation at the end of the experiment for detection of HSP 70. The brains were quickly removed and stored at 0 °C. The hippocampus, striatum and frontal cortex was dissected from the brain and placed into Eppendorf tubes. For protein extraction, the samples were weighed, rapidly thawed in 6 vols of homogenizing buffer consisting of 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride at pH 7.2, and then homogenized by a sonicator. After centrifugation at 5,000 g for 15 min at 4°C, the protein assay was carried out by the method of Lowry et al. (1951).

**Quantification of Hsp 70 by Indirect ELISA**

Prepared samples were analysed for the quantification of Hsp by Indirect ELISA. Briefly, each well of microtiter 96-well immunoassay plates (Nunc, USA) was coated with 10 μg protein per 0.1 ml samples of HSP70 overnight at 4°C, then washed with 0.05% Tween-20 in PBS (TPBS), and blocked with 0.1 ml of 2% (BSA) for 1 h at 37 °C temperature. After washing with TPBS, 0.1 ml of primary antibody (Hsp 70 antibody (5A5) Mouse monoclonal) diluted 1:4000 and added to the microtiter plates in duplicate and incubated for 2 h at 37 °C. After washing three times with TPBS, a 1:5000 dilution of peroxidase-conjugated secondary antibody (anti-rabbit IgG) in PBS (pH 7.2) was added to each well and the plates were further incubated at 37 °C for 2 h. After washing three times with TPBS, o-phenylenediamine-hydrogen peroxide in phosphate citrated buffer (pH 5.0) was added, and the color reaction developed for 3 min. The reaction was then stopped by adding 100 μl of 2.5 N H$_2$SO$_4$, and the optical density (O.D.) was measured at 490 nm with an automated microplate reader (SpectraMax 250 ELISA Reader, Molecular Devices, Palo Alto, CA, USA).

**Neurological Severity Score**

After 24 h of reperfusion, the neurological status of the animals was evaluated using two different methods: method A was used as previously described by Bederson et al. (1986). Accordingly, four categories of neurological findings were scored: 0 = no
observed neurological deficit; 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction; 2 = reduced resistance to lateral push; 3 = circling movements towards the paretic side. In method B, spontaneous motor activity (SMA) was evaluated for 5 min by placing the animals in their normal environment (cage). Neurological scoring was given as: 0 = rats moved around in the cage and explored the environment; 1 = rats moved in the cage but did not approach to all the sides and hesitated to move; 2 = rats barely moved in the cage and showed postural abnormalities (curved towards the paretic side); 3 = rats unable to move at all with their posture curved towards the paretic side.

**Histopathological studies**

Animals were sacrificed by cervical dislocation. The brain tissues were immediately removed and fixed for the histopathological studies.

**Fixation And Processing**

The brains of control and experimental groups in focal ischemia were perfused and fixed as previously described by Nakayama et al. (1989) with the mixture of formaldehyde (40%), glacial acetic acid and methanol (1:1:8, v/v). The tissues were cut into 3 mm thickness so that fixative readily penetrates throughout the tissue in short time. The tissue block was passed through a series of following solvents as per schedule for dehydration, clearing and paraffin infiltration allowing 1h at each stage. During the process of embedding, the tissue blocks were oriented so that sections are cut in desired plane of tissues followed by staining Hematoxylin and Eosin.

**RESULTS**

**Effect of Selenium pretreatment on intracellular calcium levels in synaptosomes during cerebral ischemia.**

Figure 1 shows the significantly increased (p < 0.001) levels of intracellular calcium in all 3 brain parts in MCAO group when compared with sham group. With the supplementation of selenium, a significant amelioration was seen in all the brain parts with substantial decrease in intracellular calcium levels in MCAO+S group when compared with MCAO group. In the hippocampus and striatum the significant depletion was p < 0.01 whereas in frontal cortex it was p < 0.05.

**Altered ATP levels in mitochondria during cerebral ischemia amelioration by selenium pretreatment**

Altered energy change reflected by ATP content after cerebral ischemia in 3 brain parts is shown in Figure-2. A significant decrease (p < 0.001) was seen in ATP content in MCAO group when compared with sham group. In MCAO+S group, a significant improvement (p < 0.05-0.01) was seen in hippocampus, striatum and frontal cortex respectively when compared with MCAO group.
Induction of heat stress proteins and their quantitation with ELISA during cerebral ischemia and effect of selenium pretreatment

MCAO induced heat stress proteins in MCAO group significantly (p < 0.001) when compared with sham group in the all brain parts. Whereas the induction was decreased (p < 0.01) significantly in HIP and FC in MCAO+S group when compared with MCAO group (Figure 3).

Altered behavioral outputs after MCAO and improvement offered by selenium supplementation

Cerebral ischemia induced significant changes in the behavioral assessments employed i.e., flexion test and spontaneous motor test. A marked decrease was seen (p < 0.01) in MCAO group when compared with sham. With supplementation of selenium improvement (p < 0.05) was seen in MCAO+S group when compared with MCAO group.

Caspase activation during cerebral ischemia and decreased activation after selenium supplementation

Two h of MCAO followed by 22 h of reperfusion significantly (p < 0.001) activated the caspase in the MCAO group when compared with sham group. In MCAO group supplemented with selenium a significant decrease (p < 0.01) was seen in the levels of caspase activation when MCAO+S was compared with MCAO group.
Histopathological Studies

Figure 6 is showing the histopathological changes in sham, ischemic and selenium supplemented groups.

DISCUSSION

Beneficial effects of various antioxidants in ischemic stroke have been demonstrated in a number of studies. In the present study, a significant decrement in ATP content, increase in intracellular calcium content in the synaptosomes, activation of caspases, induction of heat stress proteins and altered neurological scores were seen in MCAO group. Supplementation of sodium selenite (0.1 mg/kg, i.p) for 7 days was able to attenuate the important disrupted cellular markers of cerebral ischemia. Many studies have proven the efficacy of selenium in neurodegeneration. Likewise; Zaffar et al. (2003) from our laboratory have proved its efficacy against oxidant damage in Parkinsonism followed by Ansari et al. (2004), which evaluated its protection against oxidant damage during cerebral ischemia.

Figure 6: Showing the histopathological changes during cerebral ischemia in various groups and the protection offered by Selenium supplementation.

Slide A: High power Photomicrograph of grey matter of Hippocampal area of brain from animal in Sham operation group. Normal neurons with the characteristic conical outlines are seen (H&E 100x).
Slide B: High power Photomicrograph of tissues around infarcted area in MCAO group showing heavy concentration of microglial cells (H&E 400x).
Slide C: High power Photomicrograph of tissues in the infarcted area in Selenium + MCAO group showing edema, separation of cells with minimal microglial cell infiltration (H&E 400x).
Restoration of glutathione dependent and independent enzymatic pool which was depleted during ischemic oxidant stress was restored by selenium supplementation because it was able to inhibit mitochondrial monoamine oxidase (MAO) which is a progenitor of ROS during IR which would have otherwise deplete the antioxidant pool and lead to neuronal degeneration. In some other studies, we have observed (unpublished) that deprenyl (MAO-B inhibitor) and selenium are nonspecific quenchers of free radicals and reduce the oxidative stress.

Oxidized proteins induce Hsp 70. After cellular insult, their induction is directly correlated to levels of endogenous antioxidants, oxidized lipoproteins and protein carbonyls (Valacchi et al. 2002). Protein carbonyl content increased in MCAO group in the earlier study and selenium supplementation significantly reduced their levels afterwards. The induction of Hsp is neuroprotective and the role of Hsp70 in protecting the tissue against cerebral ischemia, especially MCAO has been investigated by many workers (States et al. 1996, Yasuda et al. 2005, Kelly and Yenari 2002). Hsp70 mRNA is not detected in the brain under normal condition (Wagstaff et al. 1996) but it increased in the ipsilateral neocortex by 1-2 h after the onset of MCAO and increases up to 24 h (Welsh et al. 1992, Kinouchi et al. 1993, Wagstaff et al. 1996, Hata et al. 2000). It is highly expressed in the ischemic boundary zone after transient or permanent MCAO (Nowak and Jacewicz 1994, Wagstaff et al. 1996), which is in accordance with the present study. An increase was observed in Hsp induction after MCAO for 2 h and reperfusion for 22 h as evidenced by ELISA. Selenium exhibited its protective effects in the ischemic boundary, a significant increase in their induction was seen in MCAO+S group in comparison with ischemic group was seen. This may be because selenium, which is already a potent effector during stressful conditions, might have induced them to provide neuroprotection. Increasing evidence that Hsp70 regulates apoptotic cell death by interfering with the functional proteins involved in apoptosis and by increasing levels of the anti death protein bcl-2, reducing the release of cyt c with subsequent DNA fragmentation thereby significantly ameliorating tissue damage (Giffard and Yenari 2004, Rajdev et al. 2000, Tsuchiya et al. 2003, Lee et al. 2001). Hsp70 may protect against ischemic death through several mechanisms, such as by preventing destruction of crucial proteins and preventing downstream stress activated kinase cascades (Taylor and Starnes 2003).

Among the molecular dysfunctions, decreased ATP content, increased intracellular calcium, activation of caspases were salvaged significantly in drug pretreated group (MCAO+S) which may be attributed to the intrinsic property of selenium to overcome oxidative stress. It reduced mitochondrial TBARS formation and most pertinent was to mask the damage to Poly (ADP-ribose) polymerase. This may be attributed to the essentiality of selenium for antioxidant enzymes, mainly selenium dependent glutathione peroxidase (Se-GPx).

PARP, a nuclear nick sensor enzyme plays a major role in DNA repair, gene expression and cell differentiation. Its cleavage during necrosis as well as in apoptotic cells leads to massive NAD+ consumption and energy depletion, which together contribute to cell death by necrosis. Selenium supplementation was able to decrease the activation of PARP, which is a latter step in cell damage, it implicates that selenium was able to restore the depleted ATP levels and increased intracellular calcium levels. In other words, selenium deficiency aggravates the apoptotic response (increased caspase activation with DNA fragmentation), probably due to impaired capacity of GPx to degrade H2O2 (Demelash et al. 2004), which is the first step of ROS quenching. Selenium inhibits caspase-3-like protease...
activity through a redox mechanism and that inhibition of caspase-3-like protease activity may be the mechanism by which selenium exerts its protective effect against UV-B-induced cell death (Park et al. 2000).

Selenium and its compounds modulate a variety of cellular activities including cell survival and death (Greeder and Milner 1980, Milner and Hsu 1981, Borak et al. 1986). Our findings in this study, that selenium suppresses caspase-3-like protease activity through a redox mechanism, may contribute significantly to a better understanding of the mechanism by which selenium participates in functions of cell survival and cell death. However, whether selenium also modulates other types of caspases besides caspase-3 with this kind of mechanism still needs to be investigated.

Park et al. (2000) reported that selenium could suppress PARP cleavage by caspase-3-like protease in vivo, pretreatment of cells with 100 nM selenium completely blocked the UV-B-induced PARP cleavage. Our data is in accordance with, which suggested the inhibition of caspase-3-like protease activity in the intact cells by selenium (Park et al. 2000). Selenium can also regulate the functions of many proteins through the oxidation of reactive cysteine residues in the proteins (Kim and Stadman 1997, Gopalkrishna et al. 1997). Those proteins that can be regulated by selenium through a redox mechanism include Na⁺,K⁺-ATPase, glucocorticoid receptor, prostaglandin synthase, nuclear factor κB and the transcription factor adaptor protein-1 (Matsumura et al. 1991, Kim and Stadman 1997).

Thus, selenium appears to regulate functions of various proteins, many of which are associated with intracellular signal transmission in diverse cellular events (McKeehan et al. 1976, Bosl et al. 1997). Selenium has a role in apoptotic machinery.

To evaluate the protection offered by selenium on neurobehavioral deficits, selenium was injected for 7 days, which reduced damage as seen in histopathological studies and improved neurologic outcomes of focal cerebral ischemia. MCAO in rats induces necrosis mainly in the frontal sensorimotor cortices and in the caudate-putamen region, which comprise a wide range of motor and sensorimotor deficits, including partial paralysis, locomotor activity, and lack of coordination, which mimic MCAO in humans (Hunter et al. 1998). The evolution of motor and sensory impairments after cerebral ischemia may considerably differ upon pharmacologic intervention (Hunter et al. 1998).

The present findings stress the importance of a separate evaluation of motor and sensory functions in the assessment of the functional benefit of potential neuroprotective agents. Supplementation of selenium before ischemic insult may considerably improve motor dysfunction after ischemic insult, which represents the major focus in the stroke rehabilitation, therefore reduce sensory deficits and alleviate cognitive and emotional impairments.