—Review—

Role of Mitochondrial Permeability Transition in the Immature Brain Following Intrauterine Ischemia

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Abstract

Recirculation following 30 minutes of intrauterine ischemia due to uterine artery occlusion has previously been found to be accompanied by delayed deterioration of the cellular bioenergetic state and of mitochondrial function in the fetal rat brain. The objective of this study was to assess whether the delayed deterioration is due to the activation of mitochondrial permeability transition (MPT), which is observed ultrastructurally as mitochondrial swelling. The respiratory activities and ultrastructure of isolated mitochondria and the cellular bioenergetic state in the fetal rat brain were examined at the end of 30 minutes of intrauterine ischemia and after 1, 2, 3 or 4 hours of recirculation. Cyclosporin A (CsA), a potent and specific MPT blocker, or vehicle was given 1 hour after recirculation. In the vehicle-treated animals, the transient ischemia was associated with a delayed deterioration of the cellular bioenergetic state and mitochondrial activities 4 hours of recirculation. The number of swollen mitochondria increased markedly after 4 hours of recirculation. Both the deterioration and swelling were prevented by CsA. The present study indicates that treatment with CsA improves recovery of energy metabolism and inhibits mitochondrial swelling following transient intrauterine ischemia in the fetal brain. The results suggest that mitochondria and MPT may be involved in the development of ischemic brain damage in the immature rat.

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Key words: mitochondria, fetal, brain, ischemia

Introduction

The developing brain is particularly resistant to hypoxic-ischemic brain injury[1–3], but the mechanisms responsible for this resistance remain unclear. Because intrauterine oxygen insufficiency is accompanied by an increase in cerebral blood flow and preferential streaming of well-oxygenated blood in the fetal heart[4], the fetal cerebrum is provided with as much oxygen as possible. However, since circulatory centralization may be partially ineffective in fetuses, brain damage may develop during severe or sustained hypoxia[5].

Recent investigations have made significant contributions to elucidating the molecular mechanisms of hypoxia-ischemia-induced neuronal injury[6]. The process starts with neuronal energy failure, which triggers a cascade of events that may culminate in either immediate or delayed neuronal

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death. Oxygen free radicals appear to play a central role in this chain of events, and mitochondria represent a target of attack[7–9]. Similar mechanisms have been implicated in perinatal hypoxic brain damage[10]. Indeed, our previous study, which examined fetal cerebral energy metabolism during intrauterine ischemia and reperfusion in rats, indicated that after ischemia an initial, partial resynthesis of ATP is followed by a secondary deterioration in cellular bioenergy [11]. This deterioration was ameliorated by a free radical spin trap agent, α-phenyl-N-tert-butyl-nitrone (PBN), which has been shown to prevent mitochondrial respiratory dysfunction due to ischemia-reperfusion injury in an adult model[8]. These findings suggest that mitochondrial activity plays a crucial role in fetal survival after intrauterine ischemia-hypoxia.

Mitochondria are important mediators in necrotic and apoptotic cell death[12]. There is extensive evidence that damage to mitochondria is a major cause of brain cell death due to ischemia and reperfusion[8,9,13–16]. The impairment of mitochondrial function is not necessarily present in the early recirculation period, but may appear after a delay, the duration of which varies with the length of the ischemic period[16–18]. In addition, the delayed dysfunction that develops in the recirculation period does not involve only a decrease in capacity for oxidative phosphorylation but also an increase in mitochondrial membrane permeability to large molecules. This event, also known as the mitochondrial permeability transition (MPT), involves the opening of proteinaceous pores in the mitochondrial inner membrane which allow the passage of ions and molecules with a molecular mass less than 1,500 Da[12,19–23]. The MPT dissipates the proton motive force, uncoupling oxidative phosphorylation, and causes mitochondrial swelling. This swelling will rupture the outer membrane of the mitochondria and cause the release of mitochondrial intermembrane proteins that can activate the initial steps of apoptosis[24–27].

Both the MPT and mitochondrial swelling are inhibited by submicromolar concentrations of the immunosuppressor cyclosporin A (CsA) and several of its structural analogues[19,28–30]. This effect appears to be mediated through the inhibition of the matrix-specific cyclophilin D[31,32], as the affinity of several CsA analogues for this protein correlates with their ability to inhibit the MPT[30]. In adult animal models, CsA, when allowed to cross the blood-brain barrier, also ameliorates damage due to forebrain or focal ischemia[33–35]. In the immature brain the effect of CsA during ischemia and reperfusion is, however, unclear.

In the cerebral cortex of immature rats, the preliminary data from our laboratory demonstrate that recirculation after transient intrauterine ischemia due to 30 minutes of uterine artery occlusion is accompanied by an initial, partial recovery of the cellular bioenergetic state and of mitochondrial function, with secondary deterioration during the first 4 hours of reflow[11,36,37]. These secondary energy failures have been described in human newborns after asphyxia[38] and in neonatal animal models[2,39–41] and reflect poor neurodevelopment[42]. Although these clinical and experimental studies implicate the MPT in perinatal hypoxic-ischemic insults, the possibility has not been investigated.

The present article presents our data on cellular bioenergetic response, mitochondrial activities, and ultrastructural signs of the MPT in the fetal rat brain during recirculation following transient intrauterine ischemia.

Animal Preparation

Maternal hypertension, chronic renovascular diseases, and several other conditions that are associated with reduced uterine blood flow during pregnancy, appear to be important pathophysiologic events in the mechanism of injury to the immature fetal brain[43]. To explore these mechanisms, maternal uterine artery ligation has frequently been used in several species of animals, including the rat. This procedure has been shown to produce fetal hypoxia, hypercapnia, and acidosis[44–46], which likely affect fetal cellular metabolism.

The experiments were performed on 10-week-old pregnant Wistar rats (Sanyo Lab Service Tokyo, Japan) weighing 250 to 300 g. The rats were housed

separately at ambient temperature with a 12-hour light cycle, and were allowed free access to water and food. On the 20th day of gestation (term 21.5 days), the animals were anesthetized with 3% halothane in a mixture of N_2O : O_2 (70 : 30) after overnight fasting. They were then intubated and artificially ventilated with 1.0% to 1.5% halothane during the operation. The tail artery was cannulated to measure arterial blood gases, pH, blood glucose, and blood pressure. A midline abdominal incision was made, and the two uterine horns were exposed and kept moist with saline. Transient uterine artery occlusion was induced according to the technique of Tanaka et al.[47]. Briefly, two microvascular clips were used to occlude the uterine vessels near the lower and upper ends of the right uterine horn. The clips were removed after 30 minutes of ischemia. During the operation core temperature was regularly maintained at 37.0℃ with a heating pad. For each experiment the fetuses in the right uterine horn served as the ischemia group and those in the left horn as the control group. After predetermined times of recovery (see below) the animals were reanesthetized, tracheotomized, and artificially ventilated. After the physiological variables had been stabilized for at least 5 minutes, fetuses were delivered by cesarean section.

Enzymatic Methods

At the time intervals chosen, corresponding to the end of 30 minutes of uterine artery occlusion or 30 minutes of uterine artery occlusion followed by 1, 2, or 4 hours of recirculation, respectively, the brain was frozen *in situ*. Cerebral cortical tissue was removed from the frozen brain at −22℃, weighed (100～200 mg), extracted with 1.0 m*l* HCl-methanol (0.1 mol/*l* HCl) and then at 0℃ with 4.0 m*l* (0.3 mol/*l*) perchloric acid, as described by Folbergrova et al[48]. The homogenate was centrifuged at 3,000 rpm for 10 minutes. The supernatant was neutralized with KOH-imidazole (1.5 M KOH, 0.4 M imidazole, 0.3 M KCl). To obtain a complete precipitation of potassium perchlorate the neutralized supernatant remained in ice water for 1 hour and was then centrifuged again at 3,000 rpm for 10 minutes. The fluorometric enzymatic techniques of Lowry and Passonneau[49] were used to measure ATP, ADP, AMP, and lactate, with analytical conditions as previously described[48].

Measurement of Tissue Oxygen Tension

In a separate series of experiments, the tissue oxygen tension was continuously monitored with a microelectrode to evaluate the blood flow changes in placental and fetal cerebral tissues throughout the whole period of 30 minutes of ischemia and 4 hour of recirculation. The both oxygen tension monitoring were performed simultaneously in a total of 6 animals.

Miniature monopolar electrodes for oxygen tension were constructed according to the method described by Rossem et al.[50] (1992). Pure platinum wire (length 15 mm; diameter 0.1 mm) was soldered to a gold connector. After being stained with black ink the wire was insulated by being dipped in cyanoacrylate glue. The measuring tip of the electrode was created manually under a microscope. First, the insulation was removed with a scalpel at the free end of the wire over a length of 0.2 mm. The naked tip was then covered with a cellulose acetate membrane by dipping it repeatedly into a cellulose acetate solution (5% cellulose diacetate in 33.3% ethanol in acetone solution).

On the 20th day of gestation, pregnant rats were kept under artificial ventilation with 0.5% to 1.0% halothane and continuous infusion of muscle relaxant (vecuronium bromide, 2 mg/h) through out the experiment. The tail artery was cannulated to measure arterial blood gases, pH, blood glucose, and blood pressure. The right uterine horn was revealed through a midline incision. Because the uterine wall and the fetal cranial vault were thin, the fetal bregma and the other superficial structures could be seen through the uterine wall. Thereafter, the microelectrode was stereotaxically implanted through the uterine wall and fetal skull for fetal cerebral tissue oxygen monitoring. The tip of the microelectrode was placed on the right side 2 mm lateral to bregma and inserted 1.5 mm below the fetal brain surface into the parietal cortex. The position of the microelectrode tip was previously

determined by anatomical observation of the fetal brain. For placental tissue oxygen monitoring, the adjoining placenta in the right horn was used. The microelectrode was carefully inserted into the placental tissue through the uterine wall (2 mm in depth). A reference electrode was placed in the upper abdominal space. After implantation, the electrode was connected to the recording circuit and polarized at −700 mV. A 15- to 30-minutes stabilization period was permitted before 30 minutes of uterine artery occlusion was started. Then tissue oxygen tensions were measured continuously throughout the entire period of 30 minutes of occlusion and 4 hours of recirculation. The changes in tissue oxygen tension are expressed as percentages of the control level.

Measurement of Mitochondrial Respiratory Function

Preparation of brain homogenates. All pups were decapitated after delivery. The whole brains were removed within 60 seconds and transferred to an ice-cold homogenization buffer containing 0.32 M sucrose, 1 mM potassium-EDTA, and 10 mM Tris (hydroxymethyl) aminomethane (Trizma Base®, Sigma Co. St. Louis, MO, USA) that had been adjusted to pH 7.4 with a pH monitor (Inter Medical, Tokyo). The forebrain was dissected out and washed free of blood with ice-cold homogenization buffer and its surface was cleared of meninges and vessels. The brains yielded 150 to 200 mg of wet tissue.

Tissue samples were homogenized in homogenization buffer using an all-glass Dounce homogenizer, according to a technique described by Sims et al.[51]. These homogenates were used to measure mitochondrial respiration. This technique measures the function of the whole population of free brain mitochondria[8,9,51].

Measurement of mitochondrial oxygen consumption. Mitochondrial respiration in prepared homogenates was measured polarographically with an oxygen microelectrode (Presearch Ltd, Basingstoke, Hampshire, UK) in a closed magnetically stirred chamber of 770-μ*l* capacity, at 28℃ (YSI Hydrodata Ltd, Letchworth, Hertfordshire, UK). Samples (usually 80～100 μ*l*) were added to the reaction buffer (usually 0.65～0.7 m*l*) containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris-phosphate, 0.05 mM potassium-EDTA, and 10 mM Tris (hydroxymethyl) aminomethane (pH 7.4). Substrates consisting of 10 μ*l* of 0.5 M glutamate and 0.5 M malate (neutralized with KOH) were also added. Stimulated (+ADP) respiration was initiated by the addition of 0.1 M ADP (typically, 0.3 μ*l* followed by 5 μ*l*). Non-stimulated (−ADP) respiration was measured from tracings obtained after the ADP added to stimulate respiration had been depleted and the rate had declined to a constant value. Uncoupled respiration was started with the addition of 5 μ*l* of 0.9 mM carbonyl cyanide *m*-chlorophenylhydrazone[8,9,51]. The respiratory control ratio (RCR) was calculated as the ratio of stimulated to non-stimulated respiration. At the conclusion of measurement, an aliquot (0.2 m*l*) was removed and frozen (−18℃) for subsequent measurement of protein content with a DC Protein Assay Kit (Bio-Rad Laboratories, CA, USA)[52].

Isolation of Forebrain Mitochondria

To isolate fetal forebrain mitochondria, all pups were decapitated immediately after delivery. The forebrain mitochondria were isolated according to the procedure of Rosenthal et al.[53] with the following modifications. The whole brains were removed rapidly, within 60 seconds, and transferred to an ice-cold isolation buffer containing 150 mM sucrose, 10 mM HEPES (pH 7.4), 1 mg/m*l* bovine serum albumin, 0.5 mM EDTA and 0.5 mM EGTA. The forebrain was dissected out and washed free of blood with the ice-cold isolation buffer, and the surface was cleared of meninges and vessels. The brains yielded 100 to 150 mg of wet tissue. Tissue samples were homogenized using an all-glass Dounce homogenizer, according to a technique described by Sims and Blass[51]. The homogenate was immediately centrifuged at 2,000 g for 3 minutes. The supernatant was decanted and centrifuged at 12,000 g for 8 minutes. The resulting supernatant was discarded and the pellet was resuspended into the isolation buffer (10 m*l*/g tissue). The suspension

was centrifuged at 12,000 g for 10 minutes. The resulting pellet was resuspended in 0.25 M sucrose (10 m*l*/g tissue) and centrifuged at 12,000 g for 10 minutes. The mitochondrial pellets were then gently rinsed with 0.25 M sucrose and suspended in 0.25 M sucrose to yield 10 to 20 mg protein/m*l*. The entire procedure was completed within 1 hour. Moreover, this mitochondrial preparation was stable for at least 3 hours with little loss of functional activity.

Fixation of Mitochondria for Electron Microscopy

Isolated mitochondrial preparations at predetermined times of recovery were ultrastructurally examined in the ischemia uterine horn. Mitochondrial suspensions were then centrifuged at 12,000 g for 10 minutes. The pellet obtained was fixed overnight with 2.5% glutaraldehyde, postfixed with 1% osmic acid in 0.1M cacodylate buffer, dehydrated, and embedded in LX-112 epoxy resin[54,55]. Thin sections were observed with a transmission electron microscope (H-7000, Hitachi Ltd, Tokyo).

To assess mitochondrial swelling, electron micrographs were analyzed at 5,000 × magnification. As described previously[56,57], a swollen mitochondria was defined as being rounded, with a diameter>0.8 μm. The number of swollen mitochondria in an area measuring 10 × 10 μm was counted, and the mean from three areas was calculated.

Cellular Bioenergetic Response to Ischemia and Reperfusion

Ishemic period. The developing brain is particularly resistant to hypoxic-ischemic brain injury, but the mechanisms responsible for this resistance are unclear. The present results demonstrate that the ATP concentration decreased to 26% of sham-operated controls at the end of 30 minutes of transient ischemia (**Fig. 1**)[11]. Recently, Kunievsky et al.[58] demonstrated that a transient and complete occlusion of the circulation of a single uterine horn was accompanied by a slow and moderate dissipation of energy levels in the fetal brain. The

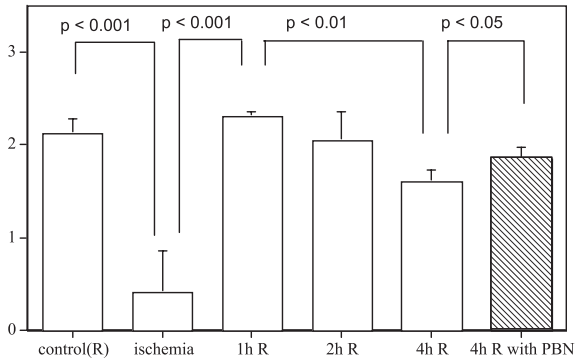

Fig. 1 After 30 min of uterine artery occlusion, the ATP concentration (nmol/kg) decreased to 26% of sham-operated controls. ATP concentration markedly improved, increasing to normal values after 1 h of recirculation. After 2 h of recirculation, ATP concentrations were maintained close to control values, but deteriorated again after 4 h of recirculation. The data on vehicle-treated animals suggest that a secondary deterioration of cellular energy state occurs after 4 h of recovery or longer.

more moderate decline in ATP levels during in utero ischemia including that in our study was consistent with the changes in high-energy phosphates in the fetal rat brain measured in vivo with ^{31}P-nuclear magnetic resonance spectroscopy[59]. A similar moderate fall in energy metabolites has been reported after short episodes of uterine blood flow arrest in the near-term guinea pigs[60]. These data from the fetal brain are in contrast to those in the adult brain in which a precipitous fall in ATP levels occurs after complete cerebral ischemia[61,62]. The slow and moderate dissipation of energy metabolites is a reflection of the resistance of the developing brain to ischemic insults and is presumably the underlying mechanism for the apparent protection. The delay in the decrease in tissue oxygen tension in the fetal cerebrum observed in the present study during the ischemic period is implicated in the slow and moderate dissipation of energy metabolites, suggestive of circulatory centralization[11].

The measured rise in cerebral lactate concentration at the end of ischemia in the present study[11] is consistent with measurements in adult animals, in which the increase in tissue lactate

concentration is the earliest sign of tissue hypoxia[63,64]. However, the rise in the cerebral concentration of lactate during hypoxemia is less in fetuses than in adults[65,66]. This difference may be due to the lower fetal cerebral metabolic rate.

Early reperfusion period. The complete restoration of ATP during the early reperfusion period (1 and 2 hours of recirculation) in the present study[11] is similar to that previously observed in a fetal hypoxia-ischemia model[58]. This complete restoration is in contrast to the partial restoration observed in models of adult ischemia, particularly with respect to ATP concentrations and the sum of nucleotides[67,68]. The present results regarding lactate, which remained high during the entire period of recirculation, are also in good agreement with previous experimental results in fetal rats and primates[58,60,69]. A more complete recovery of the lactate level has been observed in adult models of ischemia during the early reperfusion period[68]. It seems likely that the expense of anaerobic glycolysis is dominant for the maintenance of fetal cerebral energy metabolism at these time points.

Other components of the resistance to ischemia in fetuses may include the absence of postischemic hyperemia. In adult models of brain ischemia, a pronounced hyperemia occurs in the early postischemic reperfusion period[9,70,71]. In contrast, the present study, and a previous study[5], of tissue oxygen tension in fetal cerebrum indicate no hyperemia or hypoperfusion. The absence of postischemic hyperemia may be beneficial in light of observations by Kuroiwa et al.[72], who observed a decrease in tissue edema and blood-brain barrier disruption when postischemic hyperemia was prevented by constriction of the middle cerebral artery following the ischemic episode. The spontaneous limitation of postischemic blood flow in the fetus may have a similar protective effect.

Although these unique characteristics of the intrauterine environment during ischemia and reperfusion exist, secondary deterioration of the cellular bioenergetic state developed in the immature fetal brain after 4 hours of recirculation. These findings are in good agreement with the results reported by Folbergrova et al.[68] (1995), who used 2 hours of middle cerebral artery occlusion in adult rats. To explain the secondary deterioration, they suggested two possible mechanisms: secondary microcirculatory obstruction and delayed mitochondrial dysfunction. In the adult model, the first possibility seems less likely in view of reports showing an adequate return of blood flow to tissue[73] and a marked increase in tissue oxygen tension during recirculation[9,70,71]. The present results in a fetal model confirm and extend these observations because tissue oxygen tension remained unchanged during the entire period 4 hours of recirculation although secondary bioenergetic failure developed in the immature fetal brain **(Fig. 2)**[11]. This results leaves us with the likely possibility that secondary

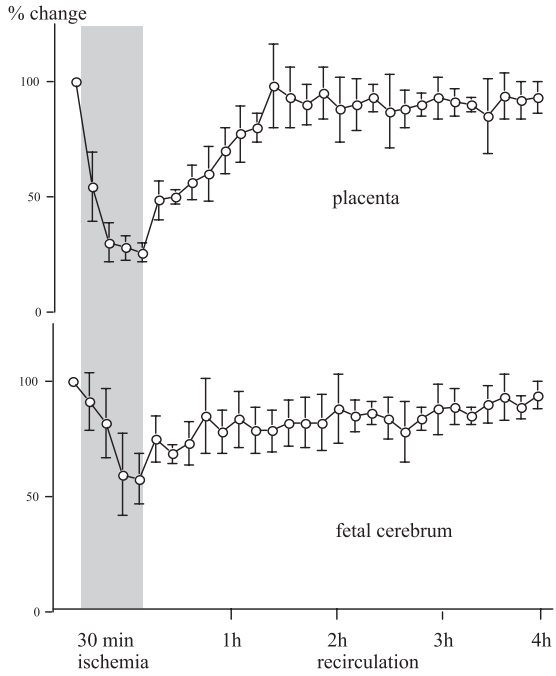

Fig. 2 Changes in tissue oxygen tension of placenta (top) and fetal cerebrum (bottom) during ischemia and recirculation. Values are presented as the means ± SD. During early ischemic period, tissue oxygen tension in fetal cerebrum showed delayed decrease compared with those in placenta. At the end of occlusion, placental and fetal cerebral tissue oxygen tension decreased to about 30% and 50% of control levels, respectively. After recirculation, the both tissue oxygen tensions increased gradually close to control values and remained almost constant throughout the whole period of 4 h of recirculation.

mitochondrial dysfunction causes bioenergetic failure.

Secondary Mitochondrial Dysfunction

In adult animals, recirculation enhances the production of reactive oxygen species that can cause membrane damage or enzyme inactivation due to lipid peroxidation or protein oxidation or both[74]. Similar mechanisms have been implicated in perinatal hypoxic brain damage. Ishimoto et al.[46] have demonstrated an increase oxygen free radicals following transient intrauterine ischemia in rats. Maulik et al.[3], who have directly measured oxygen free radicals with electron spin resonance spectroscopy, have also demonstrated similar changes in the fetal guinea pig brain.

This secondary deterioration of the cellular bioenergetic state after transient intrauterine ischemia can be explained by one of two events: secondary microcirculatory obstruction following upregulation of adhesion molecules for polymorphonuclear leukocytes[75], or delayed mitochondrial dysfunction because of oxidative stress. The first possibility seems less likely in view of reports showing an adequate return of blood flow to the fetal cerebrum and the placenta[11,46]. Indeed, in our previous study[11], the tissue oxygen tension was continuously monitored by a microelectrode to evaluate the blood flow changes in fetal cerebral tissues throughout the entire 30 minutes of intrauterine ischemia and 4 hours of recirculation. The present study has demonstrated that during ischemia tissue oxygen tension in fetal cerebrum falls to about 50% of the control level. However, after recirculation tissue oxygen tension in the fetal cerebrum increased gradually to near-control values and remained almost constant throughout the entire 4 hours of recirculation. In addition, the changes in the mitochondrial activities of the present results **(Fig. 3)**[36,76] were similar to previously reported changes in the bioenergetic state of the fetal brain[11]. This results suggests that the secondary deterioration of the cellular bioenergetic state after transient intrauterine ischemia in the immature fetal brain is due to delayed mitochondrial damage.

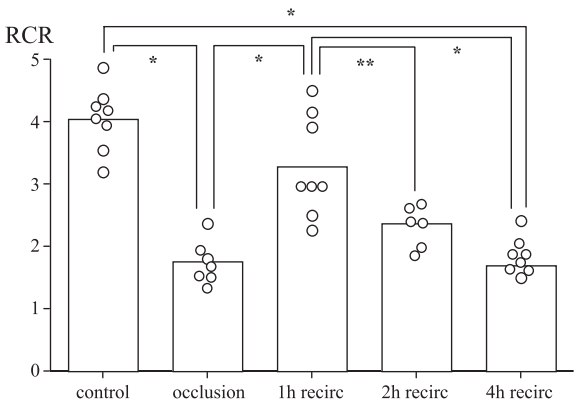

Fig. 3 Changes in respiratory control ratio (RCR) of fetal brain tissue in each group after 30 min of transient intrauterine ischemia or 1, 2, or 4 h of recirculation. Bar indicates the mean value in each group, and open circles represent the values of individual animals. Significant differences were found among the experimental groups (one-way ANOVA followed by Scheffés F test; * $p<0.001$ and ** $p<0.05$, respectively).

The mechanisms leading to mitochondrial damage are not known, but several possibilities can be considered. One is that ischemia and recirculation lead to calcium uptake by cells and mitochondria[54,77]. Accumulation of large amounts of calcium in mitochondria is potentially detrimental. First, by activating phosholipase A2, Ca^{2+} can trigger degradation of the lipid skeleton of mitochondrial membranes and cause a cascade of events, including accumulation of arachidonic acid and its break down products[78]. Some of these lipid metabolites are inhibitors (or uncouplers) of electron transport in the mitochondria, and some are formed in reactions that lead to the production of oxygen free radicals[78]. Second, by activating nitric oxide synthase (NOS), calcium can cause accumulation of nitric oxide (NO). The reaction between $^{\cdot}NO$ and $^{\cdot}O_2^-$ leads to the formation of peroxynitrite, a reactive metabolite that can nitrosylate proteins and whose composition yields a molecular species with the same chemical reactivity as $^{\cdot}OH$[79]. Indeed, our previous report demonstrated that recirculation following transient intrauterine ischemia enhances production of NO in the term fetal brain[80]. Third, calcium is a trigger of the MPT. The MPT involves the opening of a large

conductance pore in the inner mitochondrial membrane, which allows the passage of ions and molecules with a molecular mass of less than 1,500 Daltons[22]. The opening of the pore also leads to membrane changes allowing the release of proteins, increasing cytochrome C, some of which can act as transcription factors leading to gene expression and to nuclear changes observed in apoptotic cell death[24].

Role of the MPT

The present results indicate that although soon after transient intrauterine ischemia the fetal cerebral cellular bioenergetic state and mitochondrial function are maintained at close to preischemic control levels, the delayed deterioration of energy metabolism develops in the immature fetal brain. The delayed deterioration is accompanied by mitochondrial swelling that is prevented by CsA. Because pathophysiological swelling of mitochondria is associated with the activation of the MPT and because the MPT is inhibited by CsA, our data suggest that mitochondria and the MPT may be involved in the development of ischemic brain damage in the immature rat **(Fig. 4)**[81].

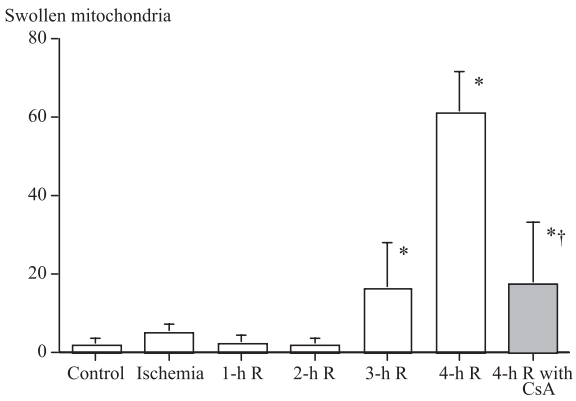

Fig. 4 Mean number of swollen mitochondria in an area measuring 10 μm × 10 μm after 30 minutes of intrauterine ischemia as well as after 1, 2, 3, and 4 hours of recirculation (R). Effects of CsA were evaluated after 4 hours of recirculation. Values are mean ± SD. * Significant difference against control (one-factor ANOVA followed by Scheffé's *F* test; $p<0.05$). † Significant difference against the vehicle treated animals at 4 h of recirculation (Mann-Whitney U test; $p<0.05$).

It has been hypothesized that long-term opening of the MPT or long-term mitochondrial swelling may cause release of apoptogenic substances such as cytochrome c from mitochondria into the cytoplasm[24–27]. Cytochrome c released from mitochondria into the cytoplasm, together with other apoptotic factors, activates caspase-3 and results in apoptosis[82]. This induction of apoptosis appears to consistent with the results of electron microscopic observation in the present study. The present results have demonstrated that although mitochondria are still compact in the vehicle-treated animals during ischemia, the number of swollen mitochondria was markedly greater after 4 hours of recirculation. These findings suggest that the delayed dysfunction of mitochondrial activities is associated with the opening of the MPT pores in this experimental paradigm. However, the results also raise the possibility that mitochondrial swelling is due to the MPT, which is believed to activate the initial step of apoptosis, because the ultrastructural changes include both apoptotic and necrotic features[83,84]. To explore this possibility, the present study examined the effects of CsA, a potent and specific MPT blocker, on the energy metabolism and the ultrastructural changes after transient intrauterine ischemia.

CsA is an effective immunosuppressant that has been used to prevent allograft rejection and, more recently, to treat auto-immune disorders. Major mechanisms of CsA include inhibition of the immune system, particularly suppression of cytokine gene expression and the activation of T lymphocytes. However, as mentioned in the introduction, the drug has more general effects of potential importance in the protection against ischemia[33–35]. Furthermore, CsA has recently been shown to ameliorate damage to mitochondria in ischemia with reperfusion[20,85]. This protective effect might be attributed to the inhibitory effect of CsA on the MPT and mitochondrial swelling[19,28–30]. On the other hand, CsA has a restricted passage into the brain parenchyma[86,87], but it accumulates to pharmacological concentrations in the brain at high blood levels[88]. Recent studies have also demonstrated a dose-dependent increase of CsA levels in the brain

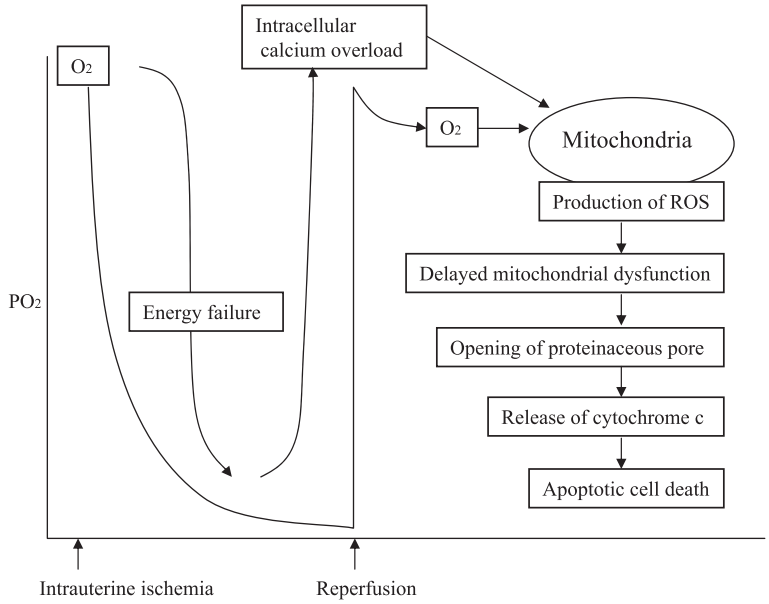

Fig. 5 Mitochondrial permeability transition in the immature brain.

parenchyma after single intravenous injections, clearly showing permeability of CsA across the blood-brain barrier at high doses[34,35,57]. In the present study, I therefore used a high dose of CsA, which is sufficient to inhibit MPT in vivo[57], and demonstrated that CsA improves recovery of the fetal cerebral cellular bioenergetic state and mitochondrial function and inhibits mitochondrial swelling following transient intrauterine ischemia, even when administered 1 hour after the start of recirculation. The protective effects are in agreement with results reported by Friberg et al.[57], who have demonstrated that CsA inhibits the MPT and mitochondrial swelling during hypoglycemic coma in vivo. Thus, the present results, which demonstrated CsA-sensitive swelling of brain mitochondria during recirculation following intrauterine ischemia, suggest that the MPT is involved in the development of ischemic brain damage in the immature rat (**Fig. 5**).

These results suggest that the delayed mitochondrial dysfunction after transient intrauterine ischemia is a trigger for apoptotic cell death, which causes the neurologic deficit apparent in neonates.

References

1. Duffy TE, Kohle SJ, Vannucci RC: Carbohydrate and energy metabolism in perinatal rat brain: relation to survival in anoxia. J Neurochem 1974; 24: 271–276.
2. Nioka S, Smith A, Mayevsky A, et al.: Age dependemce of steady state mitochondrial oxidative metabolism in the in vivo hypoxic dog brain. Neurol Res 1991; 13: 25–32.
3. Maulik D, Zanelli S, Numagami Y, et al.: Oxygen free radical generation during in-utero hypoxia in the fetal guinea pig brain: the effects of maturity and magnesium sulfate administration. Brain Res 1999; 817: 117–122.
4. Rudolph AM: Distribution and regulation of blood flow in the fetus and neonate lamb. Circ Res 1985; 57: 811–821.
5. Chao CR, Hohimer AR, Bissonnette JM: Fetal cerebral Blood flow and metabolism during oligemia and eary postoligemic reperfusion. J Cereb Blood Flow Metab 1991; 11: 416–423.
6. Siesjo BK, Siesjo P: Mechanisms of secondary brain injury. Eur J Anaesthesiol 1996; 13: 247–268.
7. Ginsberg M, Mela L, Wrobel-Kuhl K, Reivich M: Mitochondrial metabolism following bilateral cerebral ischemia in the gerbil. Ann Neurol 1977; 1: 519–527.
8. Kuroda S, Katsura K, Hillered L, Bates TE, Siesjo BK: Delayed treatment with a-phenyl-N-tert-Butyl-nitrone (PBN) atttenuates secondary mitochondrial dysfunction after transient focal ischemia in the rat. Neurobiol Dis 1996; 3: 149–157.
9. Nakai A, Kuroda S, Kristian T, Siesjo BK: The immunosuppressant drug FK506 ameliorates secondary mitochondrial dysfunction following transient focal cerebral ischemia in the rat. Neurobiol Dis 1997; 4: 288–300.
10. Digiacomo JE, Pane C, Gwiazdowski S, Mishra OP, Delivoria-Papadopoulos M: Effect of graded hypoxia on brain cell membrane injury in newborn piglet. Biol Neonate 1992; 61: 25–32.
11. Nakai A, Asakura H, Taniuchi Y, et al.: Effect of a-

phenyl-N-tert-butyl nitrone (PBN) on fetal cerebral energy metabolism during intrauterine ischemia and reperfusion in rats. Pediatr Res 2000; 47: 451–456.
12. Bernardi P, Basso E, Colonna R, et al.: Perspectives on the mitochondrial permeability transition. Biochim Biophys Acta 1998; 1365: 200–206.
13. Rehncrona S, Mela L, Siesjo B: Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. Stroke 1979; 10: 437–446.
14. Hillered L, Siesjo BK, Arfors K: Mitochondrial response to transient forebrain ischemia and recirculation in rat. J Cereb Blood Flow Metab 1984; 4: 438–446.
15. Allen K, Almeida A, Bates T, Clark J: Changes of respiratory chain activity in mitochondrial and synaptosomal fractions isolated from the gerbil brain after graded ischaemia. J Neurochem 1995; 64: 2222–2229.
16. Almeida A, Allen KL, Bates TE, Clark JB: Effect of reperfusion following cerebral ischaemia on the activity of the mitochondrial respiratory chain in the gerbil brain. J Neurochem 1995; 65: 1698–1703.
17. Sims NR, Pulsinelli WA: Altered mitochondrial respiration in selectively vulnerable brain subregion following transient forebrain ischemia in the rat. J Neurochem 1987; 49: 1367–1374.
18. Kuroda S, Siesjo BK: Reperfusion damage following focal ischemia: pathophsiology and therapeutic windows. Clin Neurosci 1997; 4: 199–212.
19. Crompton M, Ellinger H, Costi A: Inhibition by cyclosporin A of a Ca2+ dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 1988; 255: 357–360.
20. Duchen MR, Mcguinness O, Brown LA, Crompton M: On the ivolvement of a cyclosporin A sensitive mitochondrial pore in myocardial reperfusion injury. Cardiovascular Research 1993; 194: 1790–1794.
21. Richter C: Pro-oxidants and mitochondrial Ca2+: their relationship to apoptosis and oncogenesis. FEBS 1993; 325: 104–107.
22. Bernardi P, Broekemeier KM, Pfeiffer DR: Recent progress on regulation of the mitochondrial permeability transition pore: a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomemb 1994; 26: 509–517.
23. Gunter KK, Gunter TE: Transport of calcium by mitochondria. J Bioenerge Biomembrane 1994; 26: 471–485.
24. Zamzami N, Susin SA: Mitochondrial control of nuclear apoptosis. J Exp Med 1996; 183: 1533–1544.
25. Hirsch T, Marzo I, Kroemer G: Role of the mitochondrial permeability transition pore in apoptosis. Biosci Rep 1997; 17: 67–76.
26. Green DR, Reed JC: Mitochondria and apoptosis. Science 1998; 28: 1309–1312.
27. Friberg H, Connern C, Halestrap AP, Wieloch T: Differences in the activation of the mitochondrial permeability transition among brain regions in the rat correlate with selective vulnerability. J Neurochem 1999; 72: 2488–2497.
28. Crompton M, Andreeva L: On the involvement of a mitochondrial pore in reperfusion injury. Basic Res Cardiol 1993; 88: 513–523.
29. Broekemeier KM, Dempsey ME, Pfeiffer DR: Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 1989; 264: 7826–7830.
30. Halestrap AP, Davidson AM: Inhibition of Ca2+-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 1990; 268: 153–160.
31. Connern CP, Halestrap AP: Purification and N-terminal sequencing of peptidyl-prolyl cis-trans-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin. Biochem J 1992; 284: 381–385.
32. Tranveer A, Virji S, Totty NF, et al.: Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca2+ and oxidant stress. Eur J Biochem 1996; 238: 469–471.
33. Uchino H, Elmer E, Uchino K, Siesjo BK: Cyclosporin A dramatically ameliorates CA1 hippocampal damage following transient forebrain ischaemia in the rat. Acta Physiol Scand 1995; 155: 469–471.
34. Butcher SP, Henshall DC, Teramura Y, Iwasaki K, Sharkey J: Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. J Neurosci 1997; 15: 6939–6946.
35. Yoshimoto T, Siesjo BK: Posttreatment with the immunosuppressant cyclosporin A in transient focal ischemia. Brain Res 1999; 839: 283–291.
36. Nakai A, Taniuchi Y, Asakura H, et al.: Secondary mitochondrial dysfunction after transient intrauterine ischemia in the fetal brain. J Obstet Gynecol Res 2000; 26: 383–387.
37. Nakai A, Shibazaki Y, Taniuchi Y, Koshino T, Yokoyama K: Maternal treatment with NMDA receptor antagonist (MK-801) attenuates secondary mitochondrial dysfunction after transient intrauterine ischemia in neonatal rat brain. J Anesthesia 2001; 15: 217–222.
38. Azzopardi D, Wyatt JS, Cady EB, et al.: Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. Pediatr Res 1989; 25: 445–451.
39. Yager JY, Brucklacher RM, Vannucci RC: Cerebral energy metabolism during hypoxia-ischemia and early recovery in immature rats. Am J Physiol 1992; 262: H672–H677.
40. Lorek A, Takei Y, Cady EB, et al.: Delayed (secondary) cerebral energy failure after acute hypoxia-ischemia in newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr Res 1994; 36: 699–706.
41. Gilland E, Puka-Sundvall M, Hillered L, Hagberg H: Mitochondrial function and energy metabolism after hypoxia-ischemia in the immature rat brain: involvement of NMDA-receptor. J Cereb Blood Flow Metab 1998; 18: 297–304.
42. Roth SC, Edwards AD, Cady EB, et al.: Relation between cerebral oxidative metabolism following birth asphyxia, and neurodevelopmetal outcome and brain growth at one year. Dev Med Child Neurol 1992; 34: 285–295.
43. Vannucci RC, Lyons DT, Vasta F: Regional cerebral

blood flow during hypoxia-ischemia in immature rats. Stroke 1988; 19: 245–250.
44. Ogata ES, Bussey M, Finley S: Altered gas exchange, limited glucose and branched chain amino acids, and hypoinsulinemia retard fetal growth in the rat. Metabolism 1989; 35: 970–977.
45. Yavin E, Inserte-Igual J, Gil S: Ischemia-triggered translocation and inactivation of protein kinase C isoforms in the fetal brain. J Neurochem 1995; 65: 2594–2602.
46. Ishimoto H, Natori M, Tanaka M, et al.: Role of oxygen-derived free radicals in fetal growth retardation induced by ischemia-reperfusion in rats. Am J Physiol 1997; 272: 701–705.
47. Tanaka M, Natori M, Ishimoto H, et al.: Experimental growth retardation produced by transient period of uteroplacental ischemia in pregnant Sprague-Dawley rats. Am J Obstet Gynecol 1994; 171: 1231–1234.
48. Folbergrova J, Macmillan V, Siesjo BK: The effect of moderate and marked hypercapnia upon the energy state and upon the cytoplasmic NADH/NAD+ ratio of the rat brain. J Neurochem 1972; 19: 2497–2505.
49. Lowry OH, Passonneau JV: A flexible system of enzymatic analysis, 1972; Academic Press, New York.
50. Rossem K, Vermarien H, Bourgain R: Construction, calibration and evaluation of PO2 electrodes for chronical implantation in the rabbit brain cortexOxygen Transport to tissue 13, 1992; Plenum Press, New York.
51. Sims N, Blass J: Expression of classical mitochondrial respiratory responses in homogenates of rat forebrain. J Neurochem 1986; 47: 496–505.
52. Lowry O, Rosebrough N, Farr A, Randall R: Protein measurement with folin phenol reagent. J Biol Chem 1951; 193: 265–275.
53. Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S: Cerebral ischemia and reperfusion of brain mitochondrial injury by lidoflazine. J Cereb Blood Flow Metab 1987; 7: 752–758.
54. Kristian T, Gertsch J, Bates TE, Siesjo BK: Characteristics of the calcium-triggered mitochondrial permeability transition in nonsynaptic brain mitochondria: effect of cyclosporin A and ubiquinone 0. J Neurochem 2000; 74: 1999–2009.
55. Kobayashi T: The role of mitochondrial permeability transition in brain ischemia. Hokkaido J Med Sci 2000; 75: 243–252.
56. Auer R, Kalimo H, Olsson Y, Wieloch T: The dentate gyrus in hypoglycemia: Pathology implicating excitotoxin-mediated neuronal necrosis. Acta Neuropathol 1985; 67: 279–288.
57. Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP, Wieloch T: Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. J Neurosci 1998; 18: 5151–5159.
58. Kunievsky B, Pretsky A, Yavin E: Transient rise of glucose uptake in the fetal rat brain after brief episodes of intrauterine ischemia. Dev Neurosci 1994; 16: 313–320.
59. O'shaughnessy CT, Lythgoe DJ, Butcher SP, et al.: Effect of hypoxia on fetal rat brain metabolism studied in utero by 31P-NMR spectroscopy. Brain Res 1992; 551: 334–337.
60. Berger R, Jensen A, Krieglstein J, Steigelmann J-P: Cerebral energy metabolism in fetal guinea pigs during moderate maternal hypoxemia at 0.75 of gestation. J Dev Physiol 1993; 19: 193–196.
61. Nordstrom C-H, Siesjo BK: Influence of phenobarbital on changes in the metabolites of the energy reserve of the cerebral cortex following complete ischemia. Acta Physiol Scand 1978; 104: 271–280.
62. Onodera H, Iijima K, Kogure M: Mononucleotide metabolism in the rat brain after transient ischemia. J Neurochem 1986; 46: 1704–1710.
63. Siesjo BK, Nilsson L: The influence of arterial hypoxemia upon labile phosphates and upon extracellular and intracellular lactate and pyruvate concentrations in the rat brain. Scand J Clin Lab Invest 1971; 27: 83–96.
64. Siesjo BK: Brain energy metabolism, 1978; Wiley and Sons, Tronto.
65. Beck T, Krieglstein J: Cerebral circulation, metabolism, and blood-brain barrier of rats in hypocapnic hypoxia. Am J Physiol 1987; 252: H504–H512.
66. Berger R, Gjedde A, Heck J, et al.: Application of the Sokoloff method to calculate cerebral glucose consumption in fetal guinea pigs. J Cereb Blood Flow Metab 1991; 11: S869.
67. Welsh FA, Marcy VR, Sims RE: NADH fluorescence and regional energy metabolites during focal ischemia and reperfusion of rat brain. J Cereb Blood Flow Metab 1991; 11: 459–465.
68. Folbergrova J, Zhao Q, Katsura K, Siesjo BK: N-tert-a-phenylnitrone improves recovery of brain energy state in rats following transient focal ischemia. Proc Natl Acad Sci USA 1995; 92: 5057–5061.
69. Haan H, Von Reempts J, Vles J, Haan J, Hasaart T: Effect of asphyxia on the fetal lamb brain. Am J Obstet Gynecol 1993; 169: 1493–1501.
70. Nagasawa H, Kogure K: Correlation betweeen cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. Stroke 1989; 20: 1037–1043.
71. Matsuo Y, Kihara T, Ikeda M, et al.: Role of Neutrophils in radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion extracellular ascorbyl radical formation. J Cereb Blood Flow Metabo 1995; 15: 941–947.
72. Kuroiwa T, Shibutani M, Okeda R: Nonhyperemic blood flow restoration and brain edema in experimental focal cerebral ischemia. J Neurosurg 1989; 70: 73–80.
73. Tsuchidate R, He Q-P, Smith M-L, Siesjo BK: Regional cerebral blood flow during and following 2 hours of middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1997; 17: 1066–1073.
74. Floyd RA, Carney JM: Free radical damage to protein and DNA: Mechanisms involved and relevant observations on brain undergoing oxidative stress. Ann Nuerol 1992; 32: S22–S27.
75. Del Zoppo GJ, Schmid-Schobein GW, Mori E, Copeland BR, Chang CM: Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke 1991; 22: 1276–1283.

76. Nakai A, Taniuchi Y, Asakura H, et al.: Developmental changes in tolerance to transient intrauterine ischemia in rat cerebral mitochondria. Am J Obstet Gynecol 2001; 184: 731–735.
77. Kristian T, Gido G, Kuroda S, Schutz A, Siesjo BK: Calcium metabolism of focal and penumbral tissue in the rats subjected to transient middle cerebral artery occlusion. Exp Brain Res 1998; 120: 503–509.
78. Takeuchi Y, Morii H, Tamura M, Hayaishi O, Watanabe Y: A possible mechanism of mitochondrial dysfunction during cerebral ischemia: inhibition of mitochondrial respiration activity by arachidonic acid. Arch Biochem Biophys 1991; 289: 33–38.
79. Beckman J: The double-edge role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol 1991; 15: 53–59.
80. Asakura H, Nakai A, Yokota A, et al.: Continuous observation of nitric oxide production in the fetal rat brain during uteroplacental ischemia. Fetal Diagn Ther 1999; 14: 354–359.
81. Nakai A, Shibazaki Y, Taniuchi Y, et al.: Role of mitochondrial permeability transition in fetal brain damage. Pediatr Neurol 2004; 30: 247–253.
82. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell free extracts: requirement for dATP and cytochrome C. Cell 1996; 86: 147–157.
83. Wyllie AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 1980; 68: 251–306.
84. Kerr JFR, Gobe GC, Winterford CM, Harmin BV: Anatomical methods in cell death. Meth Cell Biol 1995; 46: 1–27.
85. Gogvadze V: Cyclosporin A protects mitochondria in an in vitro model of hypoxia/reperfusion injury. FEBS Lett 1993; 333: 334–338.
86. Begley DJ, Squires LK, Zlokovic BV, et al.: Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin A. J Neurochem 1990; 55: 1222–1230.
87. Shirai A, Naito M, Tatsuta T, et al.: Transport of cyclosporin A across the brain capillary endothelial cell monolayer by P-glycoprotein. Biochim Biophys Acta 1994; 1222: 400–404.
88. Bernareggi A, Rowland M: Physiologic modeling of cyclosporin kinetics in rat and man. J Pharmacokinet Biopharm 1991; 19: 21–50.

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