NEUROPROTECTIVE STRATEGY IN AN EXPERIMENTAL NEWBORN RAT MODEL OF BRAIN ISCHEMIA AND HYPOXIA: EFFECTS OF RESVERATROL AND HYPOTHERMIA

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Abstract

Introduction. *Cerebral hypoxia and ischemia are the major causes of perinatal mortality resulting in central nervous system injury. Hypoxia and ischemia produce massive brain damage and one of the most important mechanisms in the lesion pattern is oxidative stress.*

Aims. *The objectives of this study are to assess the effects of resveratrol pretreatment in a cerebral hypoxic-ischemic (HI) newborn rat model as well as the influence that hypothermia has on redox parameters in this experimental model and the effects of the combined therapy from the oxidative stress perspective.*

Material and method. *The experiment was performed on eighty newborn Wistar rats of both genders, weighing about 10 grams, placed into eight groups: control, treated with resveratrol (RES), RES+HI insult, RES+hypothermia, subjected to hypothermia only, HI insult+normothermia, HI insult+hypothermia. Resveratrol was administrated in a dose of 20 mg/kg/day for seven days as premedication. At the end of this period the animals were exposed to hypobaric hypoxia (9% O^{2+} for 90 minutes) and ischemia (by clamping the right carotid artery). In order to test the effect of combined therapy of resveratrol with hypothermia, several animals were exposed after HI injury to whole body moderate hypothermia (with 4°C) for 3h. After recovery the malondialdehyde level and the activities of superoxide dismutase and glutathione peroxidase were determined in the brain tissue of the newborn rats.*

Results. *MDA levels were increased in the groups receiving resveratrol pretreatment. In the group subjected to simple hypothermia MDA levels were also increased. In the group subjected to HI insult and then to hypothermia, MDA levels were significantly lower. MDA levels were lower also in the group where RES was associated with hypothermia in HI insult context.*

GPx levels were decreased in groups pretreated with RES and subjected to HI, hypothermia, the same effect in case of hypothermia alone. Only in the group treated with RES was the GPx level higher than in controls.

SOD levels were high in the RES pretreated group, but decreased in HI insult. In the group subjected to hypothermia after HI, SOD was significantly higher.

Conclusion. *The results of this study prove that hypothermia offers better neuroprotection in ischemic brain injuries than resveratrol. The combined therapy influenced the oxidative stress parameters. Hypothermia is a stress factor, but when applied in post-lesion condition, offers protection for the brain.*

Keywords: cerebral hypoxia-ischemia oxidative stress, resveratrol, hypothermia.

Manuscript received: 07.07.2013
Received in revised form: 25.07.2013
Accepted: 26.07.2013
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Introduction

The brain damage caused by hypoxia and cerebral ischemia is reflected in major long-term neurological morbidity and its importance is even higher when the lesions appear in an immature and intense developmental period, in a time of pronounced vulnerability as the perinatal period [1]. In view of these facts, neuroprotective strategies must be found to adequately combat the lesions that might occur. The dynamic appearance of lesions in the context of hypoxia and cerebral ischemia has been well studied. The theory of three-stage lesions is currently accepted. The first stage overlaps the first insufficient cellular energy characterized by the dramatic decrease of TPA and tissue acidosis, the second stage involves multiple pathogenic processes including the accumulation of excito-toxic neurotransmitters, oxidative stress, apoptosis, inflammation, and the third stage is the tendency of the inflammatory state to persist and the occurrence of chronic sequelae, including important developmental disorders. The target mechanism studied in this paper is the oxidative stress and how it can be influenced by the administration of resveratrol, the use of hypothermia and their combined action. Resveratrol is a natural stilbene containing three phenol groups, often in the form of trans-isomer. It is found in natural sources: grapes, hazelnuts, plums, numerous plants. One of the conditions to be used is to cross the blood-brain barrier [6].

Aims

The objective of this study was to assess the effects of resveratrol pretreatment in a cerebral HI newborn rat model, as well as the influence of hypothermia on redox parameters in this experimental model and what effects the combined therapy may have from the oxidative stress perspective.

Material and Methods

The study protocol was approved by the Ethics Committee of the University of Medicine "Iuliu Haţieganu" Cluj-Napoca (UMF) and all animal use procedures were performed accordingly. Eighty newborn Wistar rats of both genders weighing about 10 grams were used in the experiment, sourced from University's Bio-base. The pups were housed with their mothers at room temp (24+/-2°C), with 12/12h light/dark cycle. The animals were divided into the following eight groups:

Group I - control, no HI insult, no resveratrol treatment, normoxic conditions;

Group II - treated with resveratrol 20mg/kgc/d, intra-peritoneal, once a day for seven days;

Group III - treated with resveratrol then HI insult was used on post-natal 7th day (P7);

Group IV – treated with resveratrol then subjected to hypothermia for three hours on P7;

Group V - treated with resveratrol, then on P7- HI insult, then subjected to hypothermia for 3h;

Group VI – subjected to hypothermia on P7 for three hours;

Group VII – subjected to HI insult on P7, then maintained in normal thermal conditions;

Group VIII – subjected to HI insult on P7 and then to hypothermia.

In the treated groups, resveratrol was administered from the first day until P7, intra-peritoneal dose of 20 mg/kg/day. Hypoxic and ischemic insult was performed on day 7 according to the modified Levine method at the UMF Bio-base: under local anesthesia with xyline midline cervical incision was performed, isolation and ligation of the right common carotid artery. An operating microscope was used to confirm the surgical procedure. The procedure for each animal did not exceed 5 minutes. Subsequently, the animals were exposed to a hypoxic environment with 8% oxygen for 90 minutes. Then, the groups were subdivided, some being groomed under conditions of mild hypothermia maintaining intra-rectal temperature at 33-34°C, the other in normal temperature conditions for 3 hours. After being humanly put down, the brains were harvested and oxidative stress parameters were determined, evaluated for lipid peroxidations (Malondialdehyde – MDA) levels and anti-oxidant enzymes (Superoxide dismutase – SOD and Glutathione peroxidase – GPx). Measurements of oxidative stress parameters were performed in the Oxidative stress laboratory of the Department of Physiology in UMF "Iuliu Haţieganu" Cluj-Napoca.

To determine oxidative stress parameters, brain tissue was homogenized with a Polytron homogenizer (Brinkmann Kinematica, Switzerland) for 3 minutes on ice in phosphate buffered saline (pH 7.4), added in a ratio of 1:4 (w/v). MDA was determined using the fluorimetric method with 2-thiobarbituric acid described by Conti [2].

MDA was spectro-fluorimetrically determined and the values are expressed as nmoles/mg protein.

SOD activity was determined using the cytochrome c reduction test with some adjustments, according to Beauchamp and Fridovich method [3]. The results were expressed in U/g protein.

GPx activity was determined with Flohe and Gunzler method and was expressed as U/ g protein [4].

Statistical analysis

The data were expressed as the means +/- SD. Each measurement was performed in triplicate. Comparisons were made by 3- factorial ANOVA test; $p<0.05$ was considered significant.

Results

The MDA levels for neonatal rat pups brain tissue are presented in *Figure 1*. MDA levels were increased in the group receiving resveratrol pretreatment (0.36±0.06 nmoles/mg prot) comparing with the non treated group (0.28±0.12 nmoles/mg prot) in HI conditions. In the group

subjected to simple hypothermia MDA levels were also increased (0.34±0.06 nmoles/mg prot) as compared to controls (0.25±0.04 nmoles/mg prot). Only in the group subjected to HI insult and then to hypothermia were MDA levels significantly lower (0.28±0.12 nmoles/mg) respectively (0.14±0.04 nmoles/mg) p=0.01. MDA levels were lower also in the group where RES was associated with hypothermia in HI insult context (0.36±0.14 nmoles/mg prot) and 0.31±0.08 nmoles/mg prot) respectively.

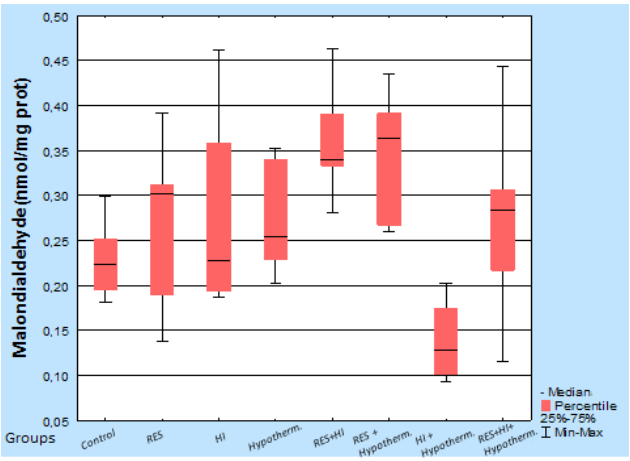

Figure 1. Neonatal rat brain tissue MDA levels.

SOD levels for neonatal rat pups brain tissue are presented in *Figure 2*. SOD levels were high in the RES pretreated group (366.72±31.69 U/g prot) compared to controls (303.4±9.91 U/g prot) p<0.001, but decreased in HI insult. In the group subjected to hypothermia after HI, SOD was significantly higher (605.24±8.9 U/g prot) respectively 462.26±4.8 U/g prot for the group subjected only to HI, p<0.001.

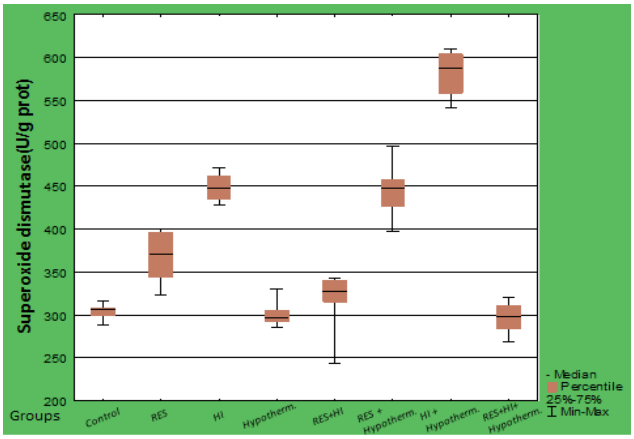

Figure 2. Neonatal rat brain tissue SOD levels.

GPx levels for neonatal rat pups brain tissue are presented in *Figure 3*. GPx levels were decreased in the groups pretreated with RES and subjected to HI, hypothermia; the same effect in case of hypothermia alone. Only in the group treated with RES was the GPx level higher than in controls (29.55±7.25 U/g prot and 24.91±8.78 U/g prot respectively, p=0.25).

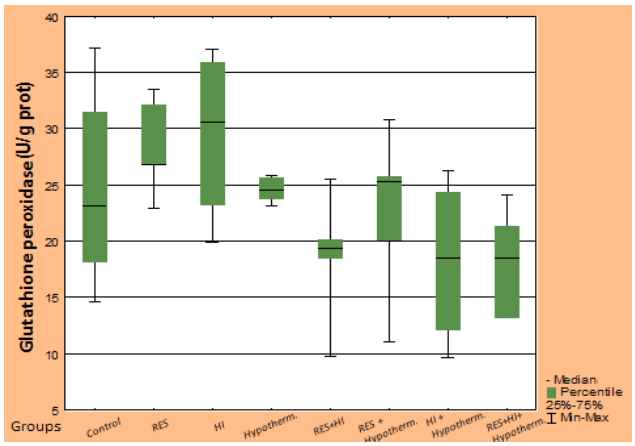

Figure 3. Neonatal rat brain tissue GPx levels.

Discussion

One of the most important links in the pathological pattern of lesions in cerebral ischemia and hypoxia is the oxidative stress [1]. Understanding this mechanism allowed the development of neuroprotective strategies to improve prognosis, and we decided to realize the experiment on newborn rats with ischemic insult on day 7, when it is generally considered that structural rat brain resembles the human newborn brain [5]. In a particular manner for the newborn, the nervous system is subjected to oxidative stress and antioxidant systems are inadequately equipped to prevent oxidative attack. There are numerous studies that have tested the effect of substances on the brain subjected to ischemia and hypoxia. In this context, we sought to evaluate the effect of both factors and strategies administered separately, and then in association. Thus we decided to administer pretreatment with resveratrol, from day one postnatal until seven days of age, when ischemia and hypoxia were induced. The efficiency of the nutritional components as neuroprotective agents is supported by empirical evidence. Plant-derived molecules, including polyphenols, demonstrated neuroprotective effect in both cell cultures and in experimental animal models. Molecular mechanisms underlying their protective actions are largely unknown. It was assumed that the effects were due to a direct antioxidant action, but low bioavailability and relatively low scavenging capacity are counterarguments [7]. A plausible explanation is that resveratrol and related polyphenols stimulates endogenous intracellular defense system, protecting against cellular stress, dysfunction and cell death, and from our results we brought another argument in support of this theory, obtaining in experimental conditions increasing of SOD after resveratrol pretreatment. The neuroprotective effects of this polyphenols were

highlighted both in cell cultures and in live animal experiments in the main parts of relevant data. In vitro resveratrol can effectively neutralize ROS by donating a hydrogen atom [9-11]. The extrapolation of in vitro results to in vivo systems is affected by numerous limitations of the experiment. In vitro studies with antioxidants often use over physiological concentrations and may be complicated by potential components to act as pro-oxidants in vitro, generating reactive oxygen species [12]. These pro-oxidant activities produce moderate oxidative stress, can induce cellular defense systems and stimulate further reactions from other stronger oxidants, similar to the preconditioning phenomenon. This phenomenon is particularly important in the context of ischemia-reperfusion injury [13]. In contrast to chemical antioxidants, resveratrol interacts with a large number of cellular systems with the potential to protect the cell. In vitro, and in vivo over-physiological concentrations of resveratrol are required for an antioxidant effect, resveratrol is a relatively weak antioxidant and scavenging properties are evident at high concentrations [14]. In addition, resveratrol and polyphenols are rapidly metabolized to glucuronide forms with reduced antioxidant capacity [15]. Despite the low probability that resveratrol act directly as an antioxidant in vivo, it seems to improve conditions associated with oxidative stress, by influencing antioxidant enzymes. One of these enzymes is mitochondrial MnSOD which converts superoxide anion to hydrogen peroxide, which is then detoxified by CAT in mitochondria and GPx into the cytosol. This enzyme is a target for resveratrol [15] and its administration in high doses increases the amount of enzyme, which could support the protective antioxidant effect of resveratrol. This mechanism has been revealed also by our study. There are fewer studies that sustained the pro-oxidant effect of resveratrol on DNA, proteins and lipids. Free radicals derived from polyphenols appear to have both pro-oxidant and antioxidant effects. Polyphenols are metabolized by peroxidases forming phenoxy-radicals which are sufficiently reactive to co-oxidate GSH or NADH, accompanied by intense takeover of oxygen and formation of reactive species. By this argument we may explain the fact that administration of resveratrol resulted in decreased GSH in all experimental conditions. Another neuroprotective factor that we have studied was hypothermia, applied after hypoxic and ischemic insult, maintaining intra-rectal temperature at 33-34°C for 3 hours, and we have obtained oxidative stress parameters decreased almost by half. Studies in animals and human patients have shown that the protective effect of mild hypothermia against cerebral lesions may be due to reduction in cerebral metabolism, inhibiting the release of excito-toxic aminoacids, immune response or attenuation changes of cell death signaling pathways. The effect of hypothermia on oxidative stress is controversial. Some authors have observed a slight increase in superoxide anion generation by mitochondria and decreased dismutation in certain types of experiments, while others have obtained a decrease in oxidative stress, using different degrees of hypothermia. For cells in general, hypothermia can be a stress factor by inhibiting the activity of enzymes that are designed to scavenge products resulting from oxidative stress. In our paper we obtained on the one hand a pro-oxidant effect of hypothermia, on normal cells without hypoxic insult; in return, when we applied hypothermia after hypoxic-ischemic lesions were produced, we obtained a spectacular protective effect against oxidative stress parameters reducing almost by half these parameters. Mild hypothermia attenuates oxidative stress by protecting mitochondrial respiratory enzymes and inducing the synthesis of MnSOD. According to some authors only mild hypothermia is confirmed as clinically effective treatment that improves the neurological outcome of patients with ischemic-hypoxic brain injury. The mechanism of action is not fully understood, but the pathogenesis of brain injury is established, being a complex cascade involving oxidative stress, glutamatergic excitotoxicity, impaired calcium homeostasis, protease cascade, activation of cell death, installed within minutes after injury and continued for 72 hours or more [17,18]. These processes are temperature-dependent, being aggravated by fever and inhibited by mild hypothermia. In this paper we studied the effect of both resveratrol and hypothermia when administered separately and the effect they have when administration was associated. Our results showed once more the protective antioxidant effect of resveratrol and suggested that hypothermia applied in patients who received resveratrol pretreatment and were subjected to hypoxic and ischemic insult had a neuroprotective effect, influencing statistically significant oxidative stress parameters.

Conclusions

The findings of the present study suggest that pretreatment with resveratrol does not offer enough protection in cerebral HI insult, but has a positive effect in non-hypoxic conditions. The most effective as a neuroprotective agent is hypothermia when used after the HI insult was produced. A positive effect appears to have the combination between resveratrol treatment and hypothermia in the HI context.

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