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Research report

Baroreceptors discharge due to bilateral aortic denervation evokes acute neuronal damage in rat brain

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ABSTRACT

Deep hypothermic circulatory arrest in cardiothoracic surgery evokes severe brain damages. On the other hand, blood pressure stimuli discontinuation to the brain has been found to induce alterations in neurotransmitter release, including glutamate, in numerous brain regions. Furthermore, it is well established that excessive glutamate release can induce neuronal injury, a process called excitotoxicity. Aim of the present study was the evaluation of possible acute neuronal damage after bilateral aortic denervation (bAD), imitating the baroreceptors discharge during circulatory arrest. Male, Wistar rats underwent either bAD or Sham operation under continuous hemodynamic monitoring. Two hours after completion of the procedure, rats were sacrificed and the brains were dissected and cut in specific levels corresponding to selective brain regions, based on either their participation in neuronal circuits, regulating blood pressure, or their vulnerability, after deep hypothermic circulatory arrest. Slices were stained and examined under light microscope using morphometric techniques. Increased number of necrotic neurons were found among bAD rats in amygdaloid complex ($p = 0.005$), motor cortex ($p = 0.001$), CA1 and CA3 ($p = 0.02$ and 0.015) but not in posterior hypothalamic nucleus and Purkinje cell. Higher ratios of necrotic neurons were found in amygdaloid complex ($p = 0.002$), motor layer ($p = 0.003$ and $p = 0.000$) and the hippocampal CA1 region ($p = 0.027$) of bAD rats. The present study shows that baroreceptors discharge due to bAD may induce acute neuronal loss in brain regions involved in blood pressure regulation. Neuronal loss might be attributed to excitotoxic phenomena and it is following the same topographic distribution seen in deep hypothermic circulatory arrest, revealing a concurrent to hypoxia/ischemia mechanism of brain damage.

1. Introduction

Deep hypothermic circulatory arrest (DHCA) is a technique frequently used concurrently for the surgical repair of complex congenital cardiac abnormalities or lesions of the aortic arch. The technique is based on temporary arrest of cardiac function (achieved with the use of cardiopulmonary bypass circuit), in conjunction with the application of profound hypothermia, in order a bloodless and still surgical field to be obtained, enabling surgical manipulations. Nevertheless, DHCA has been associated with a high incidence of transient and permanent neurologic complications, which may be attributed to the subsequent development of hypoxic–ischemic conditions [12]. Apart from well-documented development of hypoxia–ischemia and subsequent neuronal death, neuronal damage might be partially ascribed to other concurrent mechanisms [33,34]. This hypothesis is supported by the fact that DHCA-induced neuronal damage does not follow the same qualitative and quantitative regional distribution, as observed under hypoxic–ischemic conditions [6,24].

On the other hand, during the application of DHCA, patients also experienced complete temporal loss of baroreceptor input, due to the interruption of pulsatile flow and the establishment of very low static blood pressures inside the circulatory system, mainly controlled by hydrostatic factors. Previous experimental work has demonstrated complete absence of baroreceptor discharge, when static and not pulsatile arterial blood pressure is fallen in such low levels, as these, caused by circulatory arrest [8].

Many studies have focused on chronic alteration in cardiovascular function, induced by baroreceptor deafferentiation, established by bilateral denervation of the aortic depressor nerve

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(bAD) [4,16,17]. Furthermore, bAD has also been proven to induce changes in central mechanisms regulating cardiovascular homeostasis [9,20,22]. Numerous studies have recorded changes in neurotransmitter release after bAD, including glutamate and GABA, through different brain regions, involved in pressure regulation [10,14,25–28]. However, little information is available about whether or not the observed altered neurotransmitter release may induce neuronal damage, due to excitotoxicity.

Aim of the present study was to evaluate the possible acute brain neuronal damage, due to the inhibition of the afferent stimuli transmission from the aortic arc baroreceptors. For this purpose the model of experimental bAD in rats was used. Neuronal damage was investigated in areas involved either in the regulation of blood pressure, as occur for posterior hypothalamic nucleus (PH) and amygdaloid complex (AMYG), or their vulnerability, after deep hypothermic circulatory arrest. This was the case for layers III (MIII) and V (MV) of motor cortex, CA1 region of the hippocampus (CA1) and cerebellar Purkinje cells (PURK) [5,21]. The latter brain regions were chosen in order to investigate if neuronal loss in specific brain regions, observed after DHCA may be partially ascribed to excitotoxic phenomena, induced by baroreceptor input inhibition.

2. Materials and methods

2.1. Animals

Any effort was made in order to minimize pain and discomfort to the animals throughout the study. For this purpose, experiments have been conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Animal Care and Use Committee of the National Veterinary Institute and the Ethical Committee of the School of Medicine of Aristotle University of Thessaloniki.

Thirty male Wistar rats, 10–12 weeks old (230–300 g) were used. The animals were housed in a controlled environment for temperature, humidity and light (12 h light/dark cycle; lights on at 07:00). All animals had ad libitum access to food and water.

2.2. Study protocol

Under urethane anaesthesia (1.2 g/kg, i.p.) [3], animals underwent laparotomy and both left common femoral artery and vein were catheterised with two PE 50 catheters. The catheter introduced in femoral artery was connected to a multiparametric monitor (DATEX CARDIOCAP II), via a pressure transducer, in order to achieve continuous measurement of blood pressure and heart rate.

Rats were assigned into two groups to undergo either bAD (group bAD, $n = 15$), as described by Krieger [15] and modified by Singewald et al. [25], or sham operation (group SHAM, $n = 15$). In the bAD group a cervical approach was used to dissect bilaterally the area of carotid bifurcation. Bilateral cervical skin incisions were followed by muscle retraction on the level of the carotid bifurcation. The carotid bifurcation, the vagus, the aortic depressor nerve, the superior cervical ganglion and the superior laryngeal nerve were exposed; the aortic depressor nerves were bilaterally severed, just prior to their junction with the vagus. Furthermore, the superior laryngeal nerves and the superior cervical ganglia were bilaterally prepared and dissected. The incisions were closed using surgical clips. SHAM rats received similar cervical incisions and sternocledomastoids retractions, as outlined above, but the aortic depressor nerves, the laryngeal nerves and the superior cervical ganglia were neither isolated nor dissected leaving nerves, vessels and baroreceptors intact. After completion of the procedure, all rats were injected with phenylephrine (100 μl, 4 μg/kg) in order to confirm the success of aortic denervation. For this purpose, blood pressure (ΔMAP) and heart rate (ΔHR) alterations, elicited by phenylephrine, were recorded and the ratio ΔHR/ΔMAP was also calculated for each rat. ΔMAP and ΔHR are defined as the differences in MAP and HR values before and after phenylephrine administration. Animals were then kept anaesthetised for a time period of 2 h under constant conditions while mean arterial blood pressure was continuously monitored. Furthermore, arterial pressure lability, defined as the coefficient of variation (standard deviation/mean) of blood pressure values [4,14] was determined. Standard deviation for each animal was calculated from 120 measurements carried out once per minute over a period of 2 h after the completion of the procedure.

When the 2-h period had elapsed, rats were sacrificed with a lethal dose of sodium pentobarbital, while an arterial sample for arterial blood gas analysis was taken. Animals were then decapitated and the brains were fixed in 10% neutral buffered formalin, embedded in paraffin and cut in transverse slices of 4 μm thick. In every brain, representative transverse sections through the motor cortex (MIII and MV layers), hippocampus (regions CA1 and CA3), amygdala (AMYG), posterior hypothalamic nucleus (PH) and Purkinje cells of the cerebellum (PURK) were cut, according to Paxinos and Watson coordinates [19]. The slices were processed and stained with hematoxylin and eosin (H&E) for light microscopic observation.

Table 1
pO_2 values at the end of the experiment between groups.

	Number of animals	pO_2 (mean ± S.E.M.)
Group bAD	15	73.46 ± 1.49 mmHg
Group SHAM	15	73.33 ± 1.51 mmHg

2.3. Quantitative method for the measurement of necrotic neurons

The numerical density of necrotic and surviving neurons in H&E stained sections per mm^2 was estimated through the above-mentioned brain regions. Five sequential sections through each of the above brain regions were employed for the measurements in every animal of both bAD and SHAM groups. All measurements were carried out using a Zeiss Axioscope, equipped with a digital camera (SONY HyperHAD, CCP-IRIS/RGB, Colour Video Camera) and connected to a computer. The microscopic image was transferred to the computer and analyzed using specific software (Image analysis KS-300 Release 3.0). At 1000× magnification (100× objective lens, 10× eyepiece), the microscopic image, transferred to the pc screen was corresponded to an area of 0.00525 mm^2.

At a magnification of 1000×, all necrotic neurons, identified by the characteristic dense nuclei and dense eosinophilic cytoplasm and encountered within specific areas, were measured. The specific areas selected for measurements were based on previous studies, varied for different brain regions and depended on the availability of the overall area for measurements, at 1000× magnification. These areas were determined to be 0.019 mm^2 for MIII, 0.063 mm^2 for MV, PH and AMYG, 0.0063 mm^2 for CA1, 0.019 mm^2 for CA3 and 0.063 mm^2 for PURK [1]. The density of necrotic neurons per unit area examined was then transformed to the numerical density per mm^2 area of the respective brain region. For each of the brain regions examined, five different measurements were obtained, corresponded to the five sequential slices. Afterwards, the mean value of numerical density of necrotic neurons for each brain region, obtained from these five measurements, was calculated. Finally, the percentage of necrotic neurons was also counted in the same areas and the mean value of five measurements in each brain region was also computed.

2.4. Statistical analysis

Data were analyzed using statistical package, SPSS 13.0 (Chicago, IL, USA). Any differences between groups regarding MAP over time were evaluated using multivariate analysis of variance (MANOVA). ANOVAs were then conducted as follow-up tests to the MANOVA. For comparison of lability, Student's t-test for grouped data was used. Statistical analysis was carried out after logarithmic transformation. Differences between groups regarding ΔMAP, ΔHR and ΔHR/ΔMAP were analyzed using also Student's t-test.

Differences in mean values of pO_2 between groups were evaluated using unpaired t-test. Differences in mean values of numerical densities and percentages of necrotic neurons between groups were evaluated using Mann Whitney U-test. Difference between groups regarding body weight was evaluated using Student's t-test.

The level of statistical significance was set at $p < 0.05$.

3. Results

No difference was found between bAD and SHAM groups regarding body weight. Comparison between groups, regarding pO_2 values, did not reveal any difference ($p > 0.05$). The lowest pO_2 value measured was 65 mmHg for SHAM group and 67 mmHg for bAD group, respectively, indicating that none of the animals experienced hypoxia during the procedure. Mean and S.E.M. values of pO_2 are presented in Table 1.

MAP over time is presented in Fig. 1. Comparisons using MANOVA revealed significant differences between groups. Post hoc analysis showed higher MAP ($p = 0.003$), in bAD, compared to SHAM group, only in zero time point ($t = 0$). The lowest MAP limits recorded throughout the study period were 71 and 70 mm Hg, for SHAM and bAD groups, respectively.

Variations in mean blood pressure and heart rate after phenylephrine injection (ΔMAP and ΔHR, respectively) and the ratios ΔHR/ΔMAP for both groups are shown in Table 2. Phenylephrine injection induced higher ΔMAP in bAD rats, compared to SHAM operated ($p = 0.035$), while ΔHR and ΔHR/ΔMAP were significantly higher in SHAM group ($p = 0.005$).