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3 - ORIGINAL ARTICLE

GLIAL REACTION IN THE HIPPOCAMPUS AFTER GLOBAL CARDIOGENIC ISCHEMIA

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ABSTRACT: Many experimental surgerical procedures have been perfomed in the analyse of the pl brain trophism and plasticity, however undesirable intercorrence can occour leading to specific chan that should be taken into attention. To study this issue we have promoted a transient cardiogenic inte blood flow together with a transient occlusion of the bilateral common carotid arteries (2VO) in rats state of activation of astrocyte and microglia by means of the glial fibrillary acidic protein (GFAP) a immunohistochemistry, respectively. Rats were submitted to incomplete global cerebral ischemia (IC of the bilateral carotid arteries for 30 minutes. During the IGCI surgical, some rats received a higher chloral hydrate anaesthesia which promoted a cardiogenic interruption of the blood flow (CIBF) for minutes followed by and prompt reperfusion. During that period, animals were submited to a cardiac ventilated. Sham operation were made in control animals. Rats were killed and their brains processe surgery. The animals that have received a IGCI showed a slight astroglial and microglial reaction in the hippocampal formation, however the animal submitted to CIBF showed a massive infiltration of astrocyte and microglia in CA1 subfield. This results demonstrated that a transient occlusion of the b carotid arteries leads to activation of glial cells in the hippocampus, however this response can be re in animal developing a transient systemic hypoperfusion during surgery. Thus, an accurated monitor hemodinamic condition of the animal has to be done in experimental models of brain ischemia and t be analysed in view of this aspect.

SUBJECT HEADINGS: Astrocyte. Microglia. Cerebral ischemia. Imunohistochemistry. Image ana

INTRODUCTION

In the last 10 years the neuroscience field of research has been improved substantially with the devel new techniques, which have been employed in many experiments involving the study of neuronal tr plasticity. Many neurological sequeles after experimental models of neurotrauma and ischemia have be improved by neuronal trophic and plastic responses[1, 2, 3, 4, 5].

It has been showing that the paracrine trophic responses promoted by activated glial cells such as rea and microglia are substantially important in the mechanisms leading neurons to support injury as we subsequently neuronal plasticity[6, 7]. It was shown by SANTIAGO RAMON & CAJAL, DEL-RIO H many other recent investigators that the modulation in the function of reactive glial cells trigger an ic for a proper neuronal function[8]. Astrocyte and microglia readly react to neuronal lesion as well as to the neuronal homestasis[9, 10, 11, 12]. Long lasting glial reaction has also been described depending on tl magnitude of the lesion[13, 14]. Actived glial cells in an injured brain region can tigger secretion of ma which regulate the local inflamation. The later trophic and plastic neuronal events are dependent on the fashion of earlier glial reaction[6, 15, 16, 10, 17, 18].

Specific subfields of the hippocampal formation such as CA1 region have been showen to be particu ischemic damage[19]. Four vessel occlusion (4VO) model followed by reperfusion leads to a specific d CA1 subfield of the hippocampal formation three days after the insult[20]. Furtheremore, the transient occlusion of common carotid arteries (2VO) model of brain ischemia in addition with systemic hypo to injury in specific brain region[21]. It has been described that glial reaction following ischemic dama with the neuronal maintenance or degeneration[22, 23].

Many experimental manipulations like microneurosurgeries, stereotaxical injection, microdialisis ha in rodents as well as primates in order to analyse the phenomenum of brain trophism and plasticity[24] becomes necessary to demonstrate how undesirable events, i. e. systemic hypotension during neuros experimental procedures can promote specific lesions in the brain.

To study this issue we have promoted a 2VO of transient ischemia with or without cardiogenic inter flow in rats and analysed the activation of astrocyte and microglia by means of well defined markers cells such as the immunohistochemistry of the glial fibrillary acidic protein (GFAP) and OX42, resp degree of the changes was quantified by means of microdensitometric image analysis.

METHODS

Incomplete global cerebral ischemia (IGCI)

Adult male Wistar rats [body weight (b.w.) 240-280 g] from the Institute of Biomedical Science (Sã were used in the present study. The rats were kept under controlled temperature and humidity condit standardized light and dark cycle (lights on at 0700 h and off at 1900 h) and with free access to food water. Under chloral hydrate anesthesia (Merck, Germany, 0.42 mg/g, b.w.), animals were placed in apparatus and by means of a neck midline incision, the two common carotid arteries were exposed a threads without damaging the vessels and the vagus nerves. Bilateral incomplete cerebral ischemia (promoted by looping the threads wound around the common carotid arteries for 30 minutes[21, 26]. Aft reperfusion was promptly promoted.

Cardiogenic interruption of the blood flow (CIBF)

Twenty minutes after IGCI some rats received a higher dose chloral hydrate anesthesia (Merck, Ger b.w.) which promoted a cardiogenic interruption of the blood flow for a period of 10 minutes[5]. Duri

animals were submited to a cardiac massage and ventilation. Reperfusion occurs immediately after t
Thus, the total period of ICI in this groups was also 30 minutes.

Immunohistochemical procedures

Fourteen days after the global ischemia, the animals were deeply anesthetized and sacrificed by a tra
perfusion with 70 ml isotonic saline at room temperature followed by 350 ml of fixation fluid (4ºC)
minutes. The fixative consisted of paraformaldehyde in 0.1 M phosphate buffer, pH 6.9. The brains
kept in the fixative solution at 4ºC for 90 minutes, rinsed in 20% sucrose (Synth, São Paulo, Brazil)
phosphate buffered saline (PBS), pH 7.4, for 48 h, frozen in dry ice-cooled (-40ºC) isopentane (Sign
70ºC until use.

Adjacent serial 60 mm thick coronal brain sections were obtained with a cryostat (Leica, CM 3000, C
rostrocaudal levels –2.30 mm to –5.80 mm according to the atlas of PAXINOS AND WATSON[27]. T
sampled systematically during sectioning. Ten series in a rostrocaudal order including every ten sect
immunohistochemistry.

Immunoreactivity was detected by the avidin-biotin peroxidase technique[28]. Floating sections were
minutes in 0.1M PBS, pH 7.4. Sections from series one and two were used to label astrocyte and mi
respectively. The series of sections were incubated for 48 h at 4ºC under shaking with a rabbit polyc
against glial fibrillary acidic protein (GFAP, Dakoparts, Denmark) diluted 1:1200 or with a mouse n
antiserum against OX 42 (Harlan, USA) (Chadi et al., 1993; Cerutti and Chadi, 2000). The antibodie
PBS containing 0.5% Triton X-100 (Sigma) and 1% BSA (Sigma). After that, the series of the sectic
again in PBS (2 x 10 minutes) and incubated with biotinylated either goat anti-rabbit or horse anti-m
immunoglobulins, both diluted 1:250 (Vector, USA) for 2 hours. The sections were washed again in
incubated with an avidin-biotin peroxidase complex (both diluted 1:125, Vectastain, Vector, for 90 r
Immunoreactivity was visualized using 3-3'-diaminobenzidine tetrahydrocloride (DAB, Sigma) as a
H_2O_2 (0.05%, v/v, Sigma) for 8 minutes. The GFAP and OX 42 immunostained sections were count
cresyl violet to allow *interalia* the visualization of the glial cell nuclei and the neuronal cell bodies.

Semiquantitative microdensitometric analysis of the GFAP and OX42 imunoreactivities

The microdensitometric analysis was made in 3 sections on both right and left sides of the hippocam
an IBAS image analyser (Zeiss-Kontron). The subfields CA1, CA2, CA3 of the piramidal cell layers
gyrus (DG) were specifically analysed. The image analysis procedures have been described previous
the image was acquired by a television camera from the microscope (x 63.5 objective). After shadin
discrimination procedure was performed according to the following: the mean grey value (MGV) an
matter in the area of the hippocampus devoided of specific labeling (background, bg) was measured.
darker than MGV – 3 s.e.m. were considered as belonging to specific labeling and thus discriminate
(sp) MGV was then defined as the difference between the bg MGV and the MGV of the discriminat
present analysis, this parameter reflects the amount per cell of GFAP or OX42 immunoreactivities p
violet staning. It must be remembered that in the absence of a standart curve, MGV only gives semi
evaluations of the intensity of the immunoreactivity.

RESULTS

Analysis of the GFAP immunoreactivity in the sham operated rat

We found GFAP immunoreactive astroglial profiles homogeneously distributed throughout the cerel
of the neocortex of sham operated rats. In the hippocampal formation, astroglial profiles were also f
of the CA1, CA2 and CA3 subfields as well as in the DG (Fig.1A). The GFAP immunoreactive prof
moderate amount of GFAP immunoreactvity in the cytoplasm (Fig.1B). It was possible to see thin G
immunoreactive processes projecting from the citoplasm of the labeled astrocytes (Fig.1B).

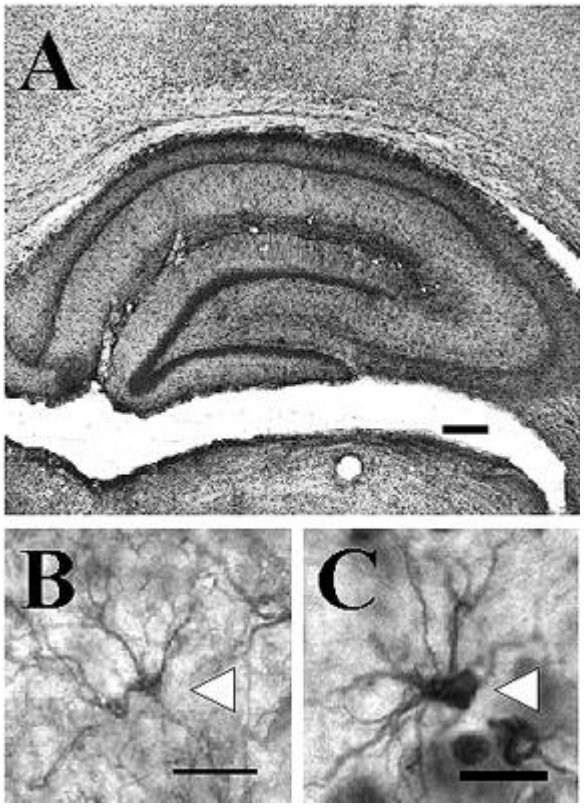

FIGURE 1

Figure 1 – Digital images showing the glial fibrillary acid protein (GFAP) immunoreactivity counterstained with cresyl violet (A-C) in the right hippocampal formation of a sham operated rat (A,B) and of an animal submitted to a transient occlusion of the bilateral common carotid arteries for 30 minutes followed by reperfusion (IGCI group, C). The animal was sacrificed 14 days after the surgery. Quiescent (B) and reactive (C) astrocytes are pointed. Barrs: 200 ㎜ (A) and 10 ㎜ (B,C).

Analysis of the GFAP immunoreactivity in the IGCI and CIBF rats

The GFAP immunoreactivity was not changed in the cerebral cortical layers of the neocortex of IGC Homogeneous distribution was also observed in all subfields (CA1, CA2, CA3 and DG) of the hippc of the ICGI rats, however a slight astroglial reaction characterized by a small increase in the number this glial cell was found in these regions of ICGI rats (Fig. 1C).

The GFAP immunoreactive profiles was also homogeneously distributed throughout the cerebral cor neocortex of the CIBF rats and they were very similar those found in that region of sham operated ra all subregions of the hippocampal formation of the CIBF rats the GFAP immunoreactive profiles ha which accumulated large amount of GFAP immunoreactive (Fig.2B). Massive increases in the numb immunoreactive astroglial profiles with enlarged cytoplasmic processes were found in the hippocam the CIBF rats (Fig.2B). Those findings were more prominent in the CA1 subfield of the hippocampa CIBF rats where a massive infliltration of reactive GFAP immunoreactive astrocytes was seen (Fig.

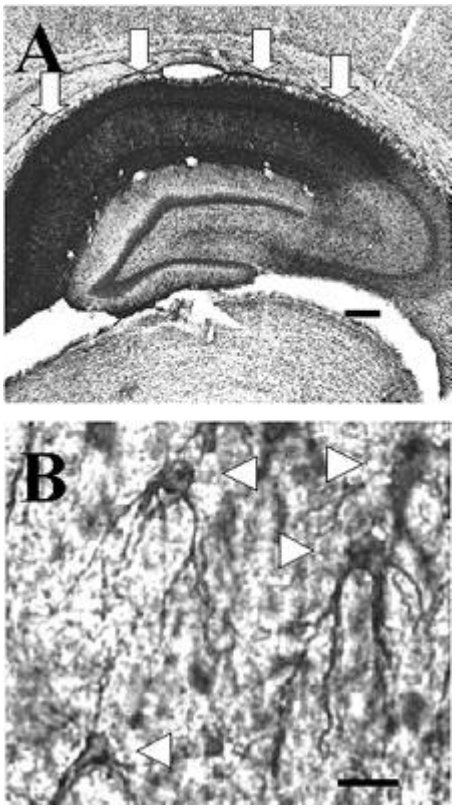

FIGURE 2

Figure 2 – Digital images showing the glial fibrillary acid protein (GFAP) immunoreactivity counterstained with cresyl violet (A-B) in the right hippocampal formantion of an animal submitted to a 20 minutes occlusion of the bilateral common carotid arteries plus 10 minutes of cardiogenic interruption of the systemic blood flow (CIBF). The animal was sacrificed 14 days after the surgery. A strong GFAP immunoreactivity is observed in the CA1 region of the hippocampus arrows (A). Reactived astrocytes are showed in the CA1 subfield of the hippocampus in B (arrowsheads). Bars: 200 mm (A) and 10 mm (B).

The microdensitometric analysis of the GFAP immunoreactivity demonstrated that the cerebral card increases the spMGV of the GFAP immunoreactive astroglial profiles by 12.52% in the region CA2 CA3 and 7.43% in the DG 14 days after the ischemic insult compared to the corespondent regions o operated (Fig.3). However, the major increase of 32% was observed in CA1 subfield of the hippocar the CIBF rats (Fig.3).

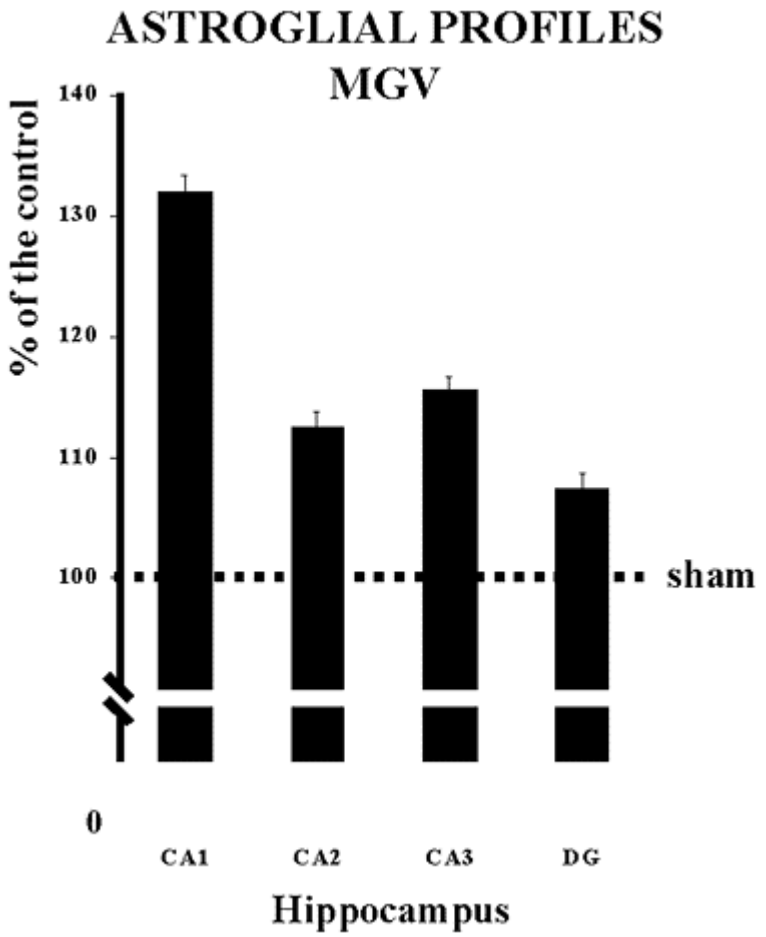

FIGURE 3

Figure 3 – Figure shows the increase of the mean grey value (MGV) of the astroglial profiles in the CA1, CA2, CA3 subregions and the denteate gyrus (DG) of the hippocampal formation of the rat submitted to a 20 minutes occlusion of the bilateral common carotid plus a 10 minutes of cardiogenic interruption of the systemic blood flow (CIBF) compared to sham operated. Astrocytes were labeled with glial fibrillary acid protein (GFAP) immunohistochemistry. Microdensitometric analysis was performed in an image analyser (for details see text).

Analysis of the OX42 immunoreactivity in the sham operated rats

We found the presence of the OX42 immunoreactive microglial profiles homogeneously distributed cerebral cortical layers of the neocortex and all subfields of the hippocampal formation of the sham OX42 immunoreactive profiles showed small cytoplasm which accumulated low amount of OX42 i (Fig.4B). It was observed several delicate OX42 immunoreactive processes projecting from the cyto

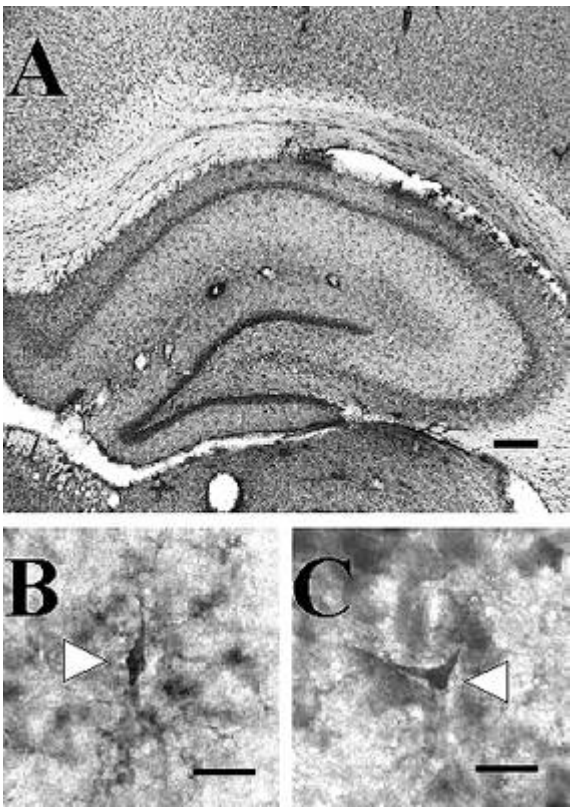

FIGURE 4

Figure 4 – Digital images showing the OX 42 immunoreactivity counterstained with cresyl violet (A-C) in the right hippocampal formation of a sham operated rat (A,B) and of an animal submitted to a transient occlusion of the bilateral common carotid arteries for 30 minutes followed by reperfusion (IGCI group, C). The animal was sacrificed 14 days after the surgery. Quiescent (B) and reactive (C) microglias are pointed. Barrs: 200 mm (A) and 10 mm (B,C).

Analysis of the OX42 immunoreactivity in the IGCI and CIBF rats

OX42 immunoreactive microglial profiles was seen homogeneously distributed throughout the cerel in the neocortex and throughout the hippocampal formation of the IGCI rats, however a slight micro characterized by an increased number of profiles could to be observed in those regions (Fig. 4C).

The OX42 immunoreactivity in the cerebral cortical layer of the neocortex of the CIBF rats was sim in the IGCI. However, an increased number of OX42 immunoreactive profiles showing enlarged cyt higher amount of OX42 immunoreactivity was found in all subregions of the hippocampal formatio (Fig. 5A). Many OX 42 immunoreactive profiles had round shape and short process in the haippoca the CIBF (Fig. 5B). A massive OX42 immunoreactivity was observed in the CA1 subfield of the hy CIBF rat (Fig. 5A). The analysis of the cresyl violet stained neuronal profiles in the subregions of th formation showed no changes in the pyramidal cell layer of the CIBF rats 14 days after surgery, how disappearance of the pyramidal neurons of the CA1 region was found after CIBF (Fig. 2A and 5A).

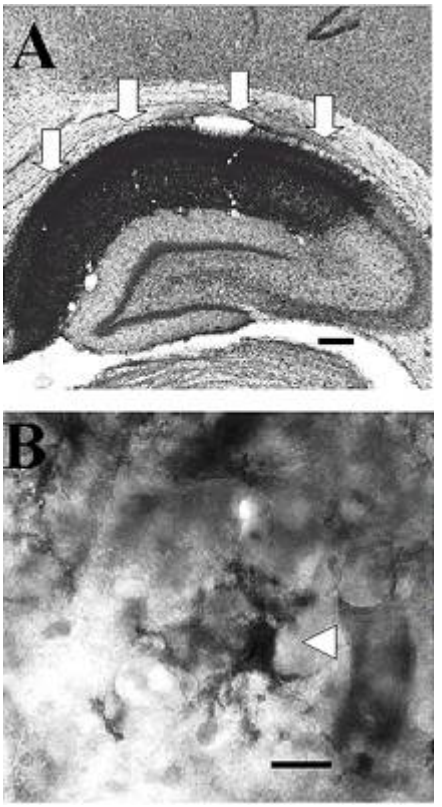

FIGURE 5

Figure 5 – Digital images showing the OX 42 immunoreactivity counterstained with cresyl violet (A-B) in the right hippocampal formantion of an animal submitted to a 20 minutes occlusion of the bilateral common carotid arteries plus 10 minutes of cardiogenic interruption of the systemic blood flow (CIBF). The animal was sacrificed 14 days after the surgery. A strong OX 42 immunoreactivity is observed in the CA1 region of the hippocampus arrows (A). Reactived microglial profiles are showed in the CA1 subfield of the hippocampus in B (arrowsheads). Barrs: 200 ㎜ (A) and 10 ㎜ (B).

The microdensitometric analysis of the OX 42 immunoreactivity demonstrated that the cerebral card increases the spMGV of the OX 42 immunoreactive microglial profiles by 9.38% in the CA2, 9.09% 2.59% in the DG 14 days after the ischemic insult compared to the corespondent regions of the sham (Fig. 6). However, the major increase of 22,21% was observed in CA1 subfield of the hippocampal f CIBF rats (Fig. 6).

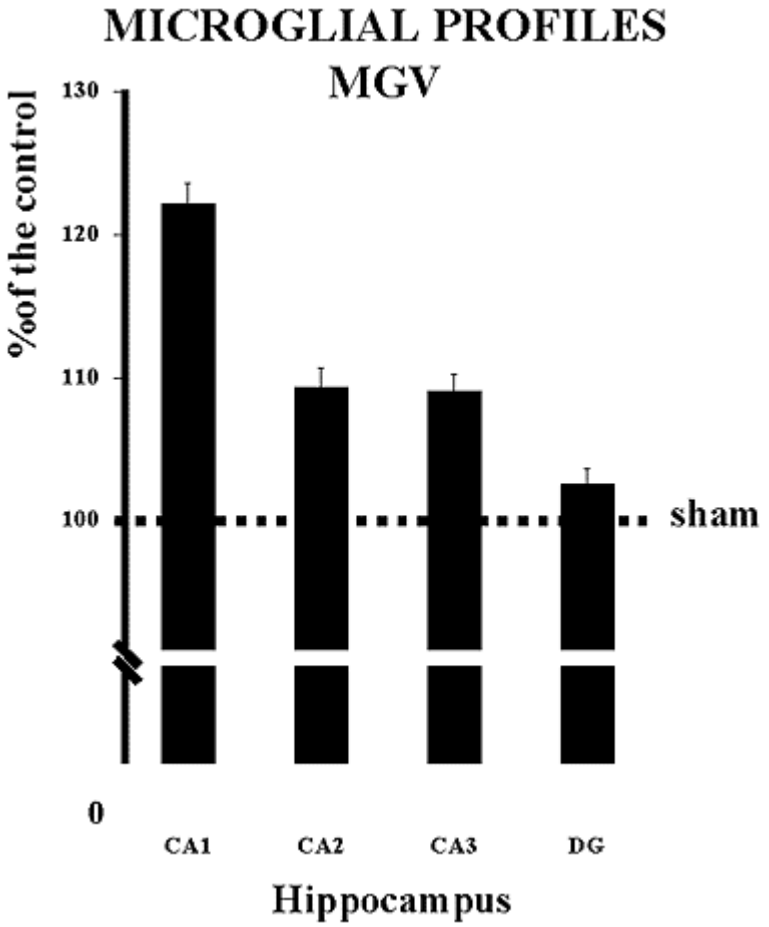

FIGURE 6

Figure 6 – Figure shows the increase of the mean grey value (MGV) of the microglial profiles in the CA1, CA2, CA3 subregions and the denteate gyrus (DG) of the hippocampal formation of the rat submitted to a 20 minutes occlusion of the bilateral common carotid plus a 10 minutes of cardiogenic interruption of the systemic blood flow CIBF compared to sham operated. Microglias were labeled with OX 42 immunohistochemistry. Microdensitometric analysis was performed in an image analyser (for details see text).

DISCUSSION

In this study an incomplete global cerebral ischemia was performed by means of a transient occlusio commum carotid arteries in a well characterized experimental model of brain hypoperfusion called 2 effects of the transient 2-VO performed here on the forebrain astroglial and microglial activation as disappearance of pyramidal neurons of CA1 region of the hippocampal formation were remarkably p temporary cardiogenic interruption of the systemic blood flow. Using the advantage of immunohisto specifically label glial cells combined with quantitative microdensitometric image analysis we have degree of glial activation in the most vulnerable brain regions to ischemia i.e. subfields of the hyppo The disappearance of neurons stained by cresyl violet was also analysed following the transient glob

Following experimental transient brain ischemia with reperfusion (IGCI procedure), morphological
neurochemical changes take place in degenerative and survival neurons as well as in the close by gli
[35, 36, 37, 38, 39]. The degree of the changes can varie depending on the resistance of a particular neuron
population[40] as well as on the locally inflammatory-mediated responses[41].

In an experimental point of view, it has also to be emphasized that regarding the effects of a transien
the hemodinamic conditions of the laboratory animals during surgery may interfere substantially wit
the results.

The 2-VO model of brain schemia in rats employed in this study has been extensively used in order
mechanisms triggering neuronal death or maintenance[21].

Another model of transient global brain ischemia called 4-VO has been also performed in rats when
lesion is desier[42]. In this model, a permanent occlusion of the vertebral arteries is followed by a trans
the common carotid arteries, bilaterally. It has also to be mentioned that the high level of mortality (
accompanied 4-VO procedure may sometimes make it difficult to elaborate more complex biochemi
experiments.

It has to be considered that anesthetic agents favorably effect outcome from brain ischemia[43] even th
be the case of chloral hydrate employed in the present work which could not prevent further damage
neurons of the hyppocampal formation after cardiogenic ischemia.

In the case of more severe ischemia followed by reperfusion, it is well known that neurons of neocor
and 6, small to medium striatal neurons and hippocampal pyramidal neurons of the CA1 and CA4 re
susceptible to schemic damaged[20, 26].

Ischemia produced by bilateral carotid artery occlusion as performed in the present analysis is able t
concentration of the extracellular amino acids glutamate, aspartate, GABA and taurune which in turn
the stimulation of adenosine A1 receptors[44]. Furthermore, a permanent occlusion of both common ca
reduces the muscanirric acetylcoholine receptor binding in the frontal cortex and hyppocampus 12 w
with learning impairment showed by the hypoperfusioned rats[45]. The heat shock protein 70 that is as
several celular processes, including DNA replication and transport of proteins across membrans, is e
CA1, CA3 and CA4 pyramidal neurons of the hippocampus following a transient forebrain ischemia
VO model[46].

It has been described that prior the death of CA1 neurons i.e. 24 hours post ischemia (four vessel occ
of the hippocampus show calpain mediated and spectrum breakdown products, an increased silver st
decreased neurophysiological response to afferent stimulation[47].

Lipid peroxidation takes place in brain regions where iron is deposited late after transient forebrain i
Because an accumulation of calcium is implicated in excitotoxic cell death, many studies have attem
the vulnerability of neurons with the presence or absence of the calcium binding proteins parvalbum
because of their calcium-buffering abilities[40].

Other fact to be considered is that the different model/intensity of brain schemia regimes may lead to
pore-like opening of the blood-brain barrier[48] which in turn may also be correlated with the selective
by changing the clearance and/or diffusion of neurotrophic and neurotoxic substances at the ischemi

A massive diminution of the pyramidal neurons stained by cresyl violet of the CA1 region of the hyp
together with a remarkable astroglial and microglial activation in this region of the CIBF rats observ
study demonstrated that the intensity of the effects promoted by the 2-VO model of ischemia may be
systemic hypoperfusion. The reduction of the mean arterial blood pressure to 40 mmHg by hypovole
has been associated with a 15 minutes occlusion of both common carotid arteries to perform a exper
incomplete cerebral ischemia[21].

The reaction of glial cells, i.e. the astrocytic response, has commonly been described following an in nervous system[14]. Animals submitted to cerebral ischemia models have showed astroglial and micro selective vulnerable brain regions[51, 49, 52]. Following a global cerebral ischemia, the insult of CA1 su hippocampal formation leads to a local infiltration of microglia and astrocyte[51, 49].

In the present analysis a higher degree of astroglial and microglial reaction was found in the CA1 su ischemic rat submitted an additional cardiogenic hypoperfusion of the blood flow, which can be corr degree of CA1 lesion, since a major disappearance of CA1 neurons was in the CIBF rats.

The upregulation of the synthesis of basic fibroblast growth factor (bFGF) by reactive astrocytes, a r factor with actions on hippocampal neurons[53] was described in the ischemic hippocampus following the other hand, reactive astrocytes can synthesize increased amount of endotelin (ET) 1 and 3 in the region after ischemia as an increased binding of ET is seen in activated microglial aggregation on da cell layer of this region[52]. These observation may help to explain the massive glial activation in the C transient global ischemia potentiated by cardiogenic hypoperfusion.

CONCLUSION

The present study demonstrated that an adequate monitoration of the hemodinamic conditions of ani experiments involving brain ischemia. Furthermore, activation of microglia and astrocytes, labeled b immunohistochemistry is a good parameter to analyse the degree of brain ischemia.

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RESUMO: Muitos procedimentos experimentais são desenvolvidos para analisar o fenômeno do trc plasticidade cerebral. Entretanto, eventos indesejáveis durante os procedimentos cirúrgicos podem o promovendo mudanças específicas nos resultados que devem ser levadas em consideração. Para estu interrupção cardiogênica transitória do fluxo sangüíneo junto com a oclussão bilateral transitória das comum (2VO) foi realizada em ratos e o estado de ativação de astrócitos e microglia foi analisado at imunohistoquímica da proteína fibrilar ácida glial (GFAP) and OX42, respectivamente. Os ratos fora isquemia cerebral global incompleta (IGCI) pela oclusão bilateral das artérias caróditas por 20 minu procedimento cirurgico da IGCI, alguns ratos receberam uma alta dose de anestésico de hidrato de c promoveu uma interrupção cardiogênica do fluxo sangüíneo (CIBF) por um período de 10 minutos. período os ratos foram submetidos a massagem cardíaca e ventilados. Uma operação simulada foi re

controles. Os ratos foram mortos 14 dias após a cirurgia e seus cérebros processados para a imunohi
animais que receberam uma IGCI apresentaram uma leve reação astroglial e microglial em todos os
formação hipocampal, entretanto os animais submetidos à CIBF mostraram uma infiltração massiça
microglia reativos no sub-campo CA1. Estes resultados demonstram que oclusão bilateral transitória
carótidas comum ativam as células gliais no hipocampo, entretanto esta resposta pode ser mudada su
nos animais desenvolvendo hipoperfusão sistêmica durante o procedimento cirúrgico. Então, monito
das condições hemodinâmicas do animal deve ser feito em modelos de isquemia cerebral e os resulta
analisados em vista deste aspecto.
DESCRITORES: Astrócito. Microglia. Isquemia cerebral. Imunohistoquímica. Analise de imagem.

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