

MILD HYPOTHERMIA REDUCES POLYMORPHONUCLEAR LEUKOCYTES INFILTRATION IN INDUCED BRAIN INFLAMMATION

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ABSTRACT - Over the last 50 years deep hypothermia (23° C) has demonstrated to be an excellent neuroprotective agent in cerebral ischemic injury. Mild hypothermia (31-33° C) has proven to have the same neuroprotective properties without the detrimental effects of deep hypothermia. Mechanisms of injury that are exaggerated by moderate hyperthermia and ameliorated by hypothermia include, reduction of oxygen radical production, with peroxidase damage to lipids, proteins and DNA, microglial activation and ischemic depolarization, decrease in cerebral metabolic demand for oxygen and reduction of glycerin and excitatory amino acid (EAA) release. Studies have demonstrated that inflammation potentiates cerebral ischemic injury and that hypothermia can reduce neutrophil infiltration in ischemic regions. To further elucidate the mechanisms by which mild hypothermia produces neuroprotection in ischemia by attenuating the inflammatory response, we provoked inflammatory reaction, in brains of rats, dropping a substance that provokes a heavy inflammatory reaction. Two groups of ten animals underwent the same surgical procedure: the skull bone was partially removed, the duramater was opened and an inflammatory substance (5% carrageenin) was topically dropped. The scalp was sutured and, for the group that underwent neuroprotection, an ice bag was placed covering the entire skull surface, in order to maintain the brain temperature between 29,5-31° C during 120 minutes. After three days the animals were sacrificed and their brains were examined. The group protected by hypothermia demonstrated a remarkable reduction of polymorphonuclear leukocytes (PMNL) infiltration, indicating that mild hypothermia can have neuroprotective effects by reducing the inflammatory reaction.

KEY WORDS: cerebral hypothermia, brain protection, brain inflammation, hypothermia.

A hipotermia moderada reduz a infiltração leucocitária na inflamação encefálica induzida

RESUMO - Nos últimos 50 anos, a hipotermia tem demonstrado ser um excelente agente neuroprotetor nas lesões isquêmicas encefálicas. A hipotermia moderada (31° C - 33° C) provou também apresentar as mesmas propriedades protetoras, sem os efeitos deletérios da hipotermia profunda. Dentre alguns mecanismos de lesão que são melhorados pela hipotermia e piorados pela hipertermia moderada, podemos citar a diminuição da demanda de oxigênio pelo encéfalo e a redução da glicina e aminoácidos excitatórios, evitando a produção de radicais de oxigênio, com aumento da peroxidase e conseqüente lesão aos lípidos, proteínas e DNA, assim como pela ativação microglial e despolarização isquêmica. Alguns estudos demonstraram que a inflamação potencializa a lesão isquêmica e que a hipotermia pode reduzir a infiltração leucocitária nas áreas isquêmicas. Para melhor elucidar os mecanismos pelos quais a hipotermia apresenta efeito neuroprotetor através da redução da inflamação, no processo isquêmico, escolhemos o método utilizando a indução de uma reação inflamatória com a utilização de uma substância com capacidade promover intensa reação inflamatória em encéfalos de ratos. Dois grupos de dez animais foram submetidos a um mesmo procedimento cirúrgico: a calota craniana foi parcialmente removida, a duramáter aberta e uma substância com potente efeito inflamatório (carragenina a 5%) foi gotejada. A pele foi suturada e, para o grupo com neuroproteção, uma bolsa de gelo foi colocada, cobrindo toda a superfície craniana, de modo a manter a temperatura encefálica entre 29,5° C e 31° C durante 120 minutos. Três dias após, os animais foram sacrificados e os encéfalos examinados. O grupo protegido pela hipotermia apresentou considerável redução na infiltração leucocitária, demonstrando que a hipotermia pode apresentar função neuroprotetora por meio de uma redução no processo inflamatório.

PALAVRAS-CHAVE: proteção cerebral, hipotermia encefálica, inflamação encefálica, hipotermia.

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In 1956 Rosomoff¹ reported that deep hypothermia (23° C) reduced ischemic damage after experimental occlusion of the middle cerebral artery in dogs. Deep hypothermia became an adjuvant method for neuroprotection in cases where circulatory arrest in complex aneurysm surgery was necessary. However the detrimental effects of prolonged deep hypothermia including delayed recovery from anesthesia, acidosis, hemodynamic compromise, blood hypercoagulability, hypotension and myocardial arrhythmia have limited the use of the technique²⁻⁴. Over the last 20 years a large number of studies have demonstrated that mild hypothermia (31-33° C) can have the same neuroprotective effect provided by deep hypothermia in ischemic brain^{3,5-11}. The mechanisms underlying this neuroprotection have been attributed to decrease in cerebral metabolic demand to oxygen and reduction of glycine and excitatory amino acid (EAA) release. Glutamate release occurs 1-5 h after ischemic onset and mild hypothermia can be protective even if delayed by 2 hours¹². Corbett et al.¹⁰ demonstrated that delayed hypothermia reduces focal ischemic injury. Therefore, although reducing EAA release⁵ and glycine and glutamate release¹², mild hypothermia can have other neuroprotective effects other than EAA, glutamate and glycine release. Even more, while neuroprotection by deep hypothermia can be explained by a decrease in cerebral blood flow and metabolic demand for oxygen, this by itself cannot fully explain the equally protection that has been shown when the temperature is lowered by only a few degrees^{13,14}. A high degree of neuroprotection was conferred by postischemic cooling (2h) to 32° C which is virtually equivalent to that observed with intras ischemic cooling at the same level in focal cerebral ischemia¹⁴.

It has been documented that inflammation contributes significantly to cerebral injury following ischemia^{7,15}. Inflammatory cells presumably promote ischemic cell damage by microvascular occlusion. This may prolong and intensify the ischemic event¹⁶. Cytotoxic inflammatory reactions caused by microglial activation and blood-borne neutrophils have been implicated in the pathogenesis of ischemia/reperfusion brain injury^{2,3,14,17-19}. Neutrophils began to infiltrate into an infarct area soon after ischemia. Cytokine expression may be the earliest sign of the inflammatory response. Cytokines activate microglia and stimulate expression of endothelial adhesion leading to leukocyte infiltration. PMNL

accumulation is maximal at 48-72h. Attenuation of the inflammatory response may be one of the mechanisms by which hypothermia reduces ischemic neuronal injury^{13,19,20}.

Since it has been demonstrated that carrageenin is a highly reactive substance with inflammatory properties²¹ and brain inflammation can be induced with its subarachnoidal injection in brains of mice²², in this study we aimed to demonstrate that mild hypothermia can have neuroprotective effects in brains of rats submitted to inflammatory injury by means of the use of carrageenin.

METHOD

Animal protocols were approved by the Federal University of S. Paulo animal's ethic board. Institutional guidelines were followed in all protocols. All animal experiments were conducted in accordance with the NIH guide for the care and use of laboratory animals (NIH publication 80-23). All efforts were made to minimize animal suffering, and only the smallest number of animals were used to generate reliable scientific data.

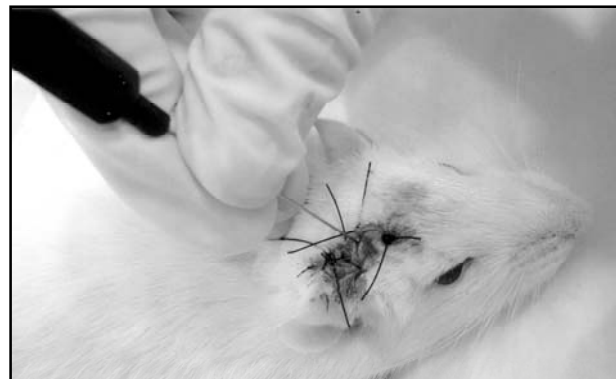


Fig 1. Line of incision of the skin.



Fig 2. Brain temperature is observed. Probe is inserted 5mm within the brain parenchyma.

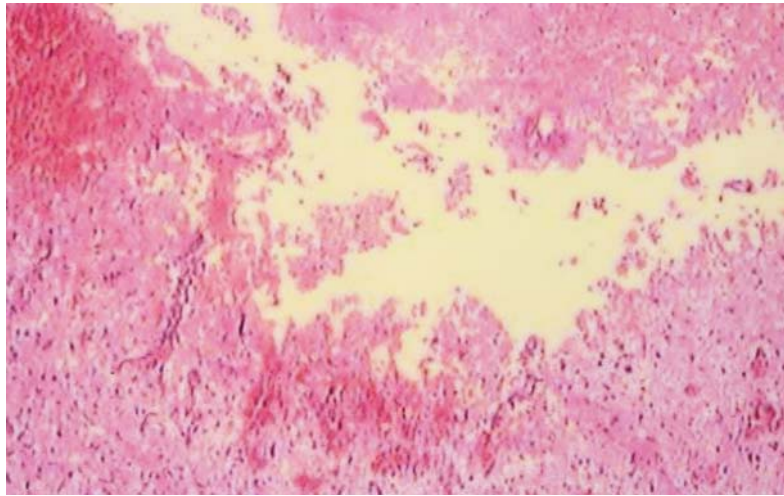


Fig 3. Brain of rat. Haematoxylin-eosin 200X. Slices obtained three days after 5% Carrageenin was dropped. No neuroprotection was performed. Small necrotic area and marked inflammatory infiltration can be seen.

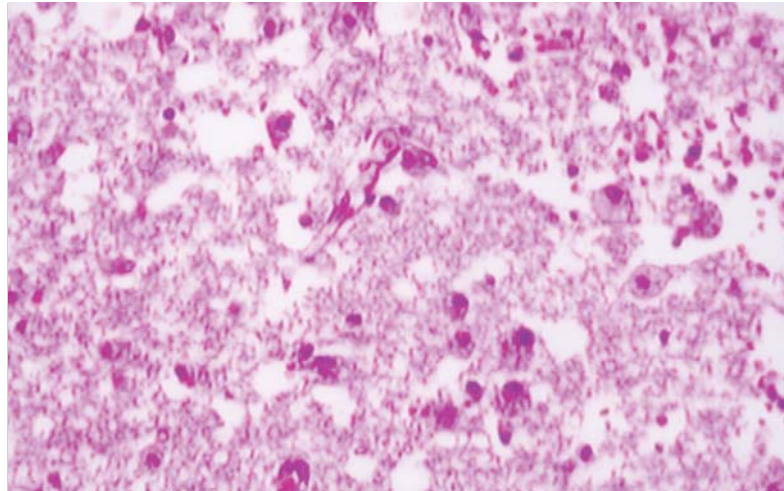


Fig 4. Brain of rat. Haematoxylin-eosin 200X. Slices obtained three days after 5% Carrageenin was dropped. Neuroprotection with hypothermia (30° C) for 120 minutes. Macrophage infiltration with capillary proliferation can be seen.

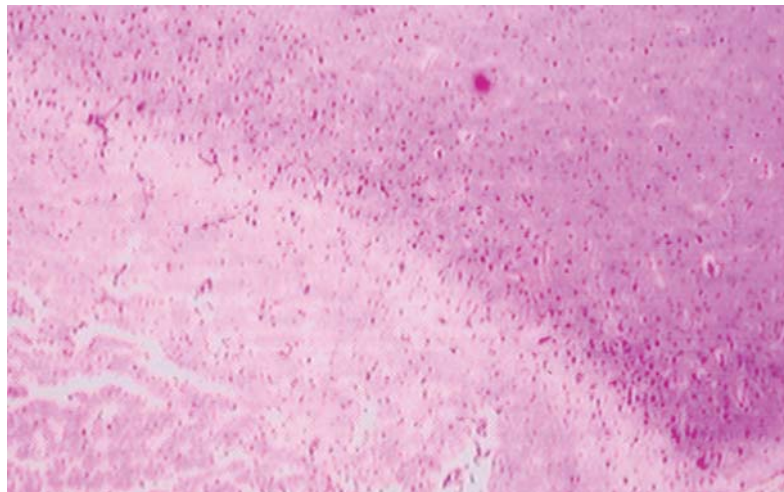


Fig 5. Brain of rat. Haematoxylin-eosin 40X. Slices obtained three days after 5% Carrageenin was dropped. Normal and pathological areas can be seen.

Table 1. Total number of PMNL in group 2 and 3.

PMNL/FIELD	GROUP 2	GROUP 3
1.	98	30
2.	88	38
3.	96	28
4.	72	24
5.	80	38
6.	72	32
7.	88	34
8.	72	40
9.	88	24
10.	96	32

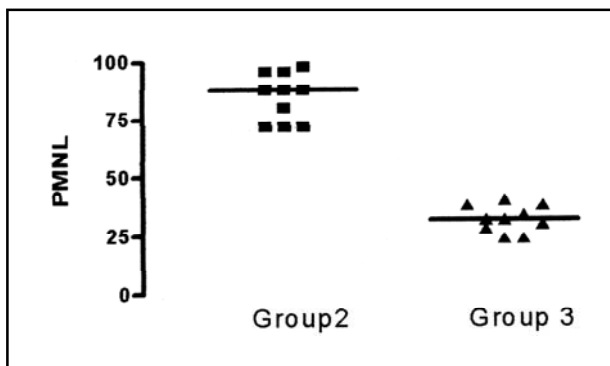


Fig 6. Scatter plot of PMNL accumulated after topical Carrageenin application.

Three groups of ten Wistar E.PM rats weighing between 290g and 330 g were studied.

Group 1 - control group, Group 2 - received no protection by hypothermia and; Group 3 - received protection by hypothermia.

General anesthesia was given by means of IM injection of tiletamin chloridrate + zolazepan chloridrate in the proportion of 100mg/kg. The animals were placed in a specially designed table covered by an homeothermic blanket control unit (Harvard Apparatus Limited Cat 50-7079 Edenbridge Kent). The core temperature was maintained in 37°C and was measured with a flexible fiber with the sensor tip placed into each animal's rectum (El-lab medical precision thermometer DM 852). The scalp incision measuring 18mm was C shaped 4mm from the midline (Fig 1). The muscles and fascia were dissected from the cranium. Under an operating microscope a 10-12 mm burr hole was drilled and the dura was exposed. After the duramater had been incised, 2-3 drops of solution of 5% beta-carrageenin was topically dropped. The scalp was sutured with mononylon 3-0. In the group 3, (the one protected by hypothermia), an ice bag was pla-

ced in order to cover the entire head. The ice bag was removed whenever the brain temperature dropped below 29,5°C and replaced when the temperature reached 31°C. Brain temperature was measured with a needle probe placed subcutaneously inserted 5mm into the brain parenchyma (Fig 2) (El-lab medical precision thermometer DM 852). The hypothermia was maintained for 120-130 minutes. The control group underwent the same surgical procedure but no inflammatory solution was dropped, nor was hypothermia performed.

After 3 days the animals were anesthetized and sacrificed. All bone of the superior part of the skull was withdrawn in order to permit the removal of the whole brain that was immediately fixed in formalin 10,0%. The specimens were allowed to fix for 24 hours, and then embedded in paraffin.

Histopatological examination – Brains were sliced into 18-µm-thick coronal sections and stained with hematoxylin and eosin with magnification X40, X100, X200. High microscopy examination aimed to demonstrate the number of PMNL per field. Four fields were examined. Total number of cells was counted using original magnification X 200. Histopatological analysis revealed acute lymphocytic and macrocytic cell predominance; small areas of necrosis were also seen (Fig 3) in some cases without neuroprotection. Inflammatory infiltration was only seen in the specimens where carrageenin was dropped (Fig 4). In brain hemisphere that has not received carrageenin (Group 1) no significant sign of inflammatory reaction was identified. Normal and pathological areas can be seen in Figure 5. In group 2 and 3, the total number of PLNL is demonstrated in the Table 1.

Statistical analysis – Data are presented as means \pm standard deviation. They were compared by analysis of variance followed by Mann Whitney Test. Data were considered different at $p < 0.5$ (Fig 6).

DISCUSSION

Rosomoff¹ first reported that deep hypothermia reduced ischemic damage after experimental occlusion of the MCA in dogs. The use of deep hypothermia has been limited because of several undesirable side effects including acidosis, blood hypercoagulability, delayed recovery from anesthesia, hemodynamic compromise, myocardial arrhythmia and hypotension²⁻⁴. More recently several studies have shown that mild hypothermia can have the same neuroprotective effect in ischemic models using rodents, cats and dogs and the severe limitations associated with deep hypothermia can be avoided⁵⁻¹⁰. Prandini et al.¹¹ have demonstrated that mild hypothermia, locally produced in rabbit's brains, can redu-

ce infarct size in cases of ischemia produced by coagulation of the middle cerebral artery.

It has been a matter of debate the mechanisms implicated in the neuroprotective effects of hypothermia. A large number of studies have shown that the mechanisms of neuroprotection conferred by moderate hypothermia are multifactorial^{5,14-16}. Mechanisms of injury that are ameliorated by hypothermia and exaggerated by moderate hyperthermia include, oxygen radical production, with peroxidase damage to lipids, proteins and DNA, microglial activation and ischemic depolarization^{5,14-16}, decrease in cerebral metabolic demand for oxygen and reduction of EEA and glycerin release¹² also cannot fully account for the neuroprotective effect of hypothermia. Recent studies have demonstrated that inflammation is a major determinant of neural death following ischemia^{15,17-19,24}. Focal ischemia and reperfusion of the neocortex elicit a substantial cell-mediated inflammatory response and produced cell infarction²³⁻²⁵. Cellular inflammation is initiated by ischemia at the blood microvascular endothelial cell interface¹⁴.

Polymorphonuclear leukocytes are early participants in the cerebral microvascular response to focal ischemia^{16,18}. The presence of PMNL in occluded microvessels within 60 minutes after MCA occlusion in baboons has been demonstrated¹⁵. Developing infarction was accompanied by accumulation of inflammatory cells of both intrinsic (microglia) and extrinsic (macroglia) origin²⁵. Treatment with anti-pyretic and anti-inflammatory drug dipyron delays neuronal damage in the rat hippocampus, cortex and striatum²⁶. Anti-adhesion molecule antibodies selectively reduces apoptosis in transient middle cerebral artery in rat brain²⁷. Toyoda et al.²⁴ demonstrated that intraschemic hypothermia attenuates neutrophil infiltration in the rat neocortex after focal ischemia-reperfusion-injury. Kawai et al.¹³ have demonstrated that ischemic brain damage can be reduced with delayed hypothermia and prolonged postischemic hypothermia in a focal model of transient cerebral ischemia in rats. One of the mechanisms by which hypothermia reduces ischemic neuronal injury is by attenuating the inflammatory response^{13,19}. Intraischemic hypothermia reduced the volume of infarction by 59% compared with the normothermic animals. Since the accumulation of PMNL is maximal at 48-72h after ischemic insult, our specimens were obtained only 72 h after the inflammatory reaction had begun.

While several studies have begun to elucidate the contribution of the inflammatory response to cerebral ischemic injury, the effect of hypothermia on the inflammatory response is still unclear^{7,10,15,17-19}.

Carageenin was used in our experiments because for more than 50 years it has been considered as one of the best drugs to produce inflammatory activity²¹ and its inflammatory properties when subarachnoidally injected have been demonstrated²². Our results indicate that mild hypothermia effectively reduced the leukocyte infiltration on brains of rats subjected to this potent inflammatory substance. This could explain one additional mechanism of protection provided by mild hypothermia.

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