Recruitment of neutrophils across the blood-brain barrier. The role of posttraumatic hepatic ischemia[1]

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Mantovani M, Fontelles MJ, Hirano ES, Morandin RC, Schenka AA. Recruitment of neutrophils across the blood brain barrier: the role of posttraumatic hepatic ischemia. Acta Cir Bras [serial online] 2003 Sept-Oct;18(5). Available from URL: http://www.scielo.br/acb.

ABSTRACT – Purpose: To study the effects of total hepatic ischemia, and reperfusion on the accumulation of neutrophils in the brain of rats submitted to normovolemic conditions as well as to controlled hemorrhagic shock state. **Methods**: Thirty two adult male Wistar rats, were divided into four groups: the Control group, was submitted to the standard procedures for a period of 60 min of observation; Shock group, was submitted to controlled hemorrhagic shock (mean arterial blood pressure=40mmHg, 20min) followed by volemic resuscitation (lactated Ringer's solution + blood, 3:1) and reperfusion for 60min; Pringle group, was submitted to total hepatic ischemia for 15min and reperfusion for 60min. The total group was submitted to controlled hemorrhagic shock for 20min followed by volemic resuscitation (lactated Ringer's solution + blood, 3:1), total hepatic ischemia for 15min and reperfusion for 60min. Measurements of serum lactate and base excess were used to characterize the hemorrhagic shock state with low tissue perfusion. The counting of neutrophils on the brain was performed after the euthanasia of animals. **Results**: The values for the counting of neutrophils on the brain indicate that did not occur difference among studied groups (p=0.196) (Control 0.12±0.11, Shock 0.12±0.13, Pringle 0.02±0.04, Total 0.14±0.16). **Conclusion:** Hemorrhagic shock associated to total hepatic ischemia for 15 minutes, followed by 60 minutes of reperfusion, did not causes significant neutrophils accumulation in the brain of rats.

KEY WORDS – Blood-brain barrier. Hemorrhagic shock. Pringle's maneuver. Neutrophils.

Introduction

Trauma is one of the main causes of death among the adult population in general – running the third place, and first until the age of 44 years. The liver is amongst the hit organ in the abdominal trauma[1]. These patients suffering severe liver damage often develop hemorrhagic shock, then it may be followed by an inflammatory generalized response with intense systemic compromise, and pulmonary involvement – the Adult Respiratory Distress Syndrome (ARDS) – or the final involvement of multiple organs, known as the Multiple Organ Failure – MOF[2,3].

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The surgical approach in these patients is to stop bleeding by occlusion of the hepatoduodenal ligament as described by Pringle in 1908. In spite of the fact, that this maneuver is easy to perform, fast and efficient, it cause total transient ischemia of the liver, and may lead to important histologic changes that may worsen the recovery during the volemic replacement phase (reperfusion), due to substances that triggers the inflammatory response, as it is the case with cytokines and the migration of neutrophils to the tissues, such as an human model of ischemia and reperfusion (I/R)[4]. In this manner, there are controversies if overactivation of leukocytes alter the blood-brain barrier (BBB) permeability during hemorrhagic shock following severe hepatic trauma.

Thus, the purpose of the present work was to study the effects of total hepatic ischemia and reperfusion on the accumulation of neutrophils in the brain of rats submitted to normovolemic conditions as well as to controlled hemorrhagic shock state.

Methods

All the procedures undertaken in this work comply with the protocols approved in the Ethic Commission on Animal Experiment of the Biologic Institute of the University of Campinas, Brazil.

Animals – 32 Wistar rats (UNICAMP, Campinas, SP) with body weight ranging from 185g to 250g (212.85±20.79) and eight weeks of age, all animals were kept under controlled condition of lighting and adapted to experimental environment.

Grouping – These animals were randomly distributed in four groups as described below:

- Control group – Normal rats subjected only to laparotomy followed by a period of observation lasting 60 minutes, the same period of reperfusion used in the other groups.
- Shock group – Rats bled until a shock stage developed, the mean arterial blood pressure (MAP) kept at 40mmHg for 20 minutes, then the volemia was corrected by infusion of lactated Ringer's solution and blood (3:1 proportion) until the mean arterial blood pressure (MAP) reached 80mmHg and followed by additional period of reperfusion for 60 minutes.
- Pringle group – These rats had a laparotomy performed followed by 15 minutes of Pringle's maneuver then total liver reperfusion for 60 minutes.
- Total group – Rats subject to bleeding until a state of hemorrhagic shock was attained with the MAP held at 40mmHg for 20 minutes then a volemic replacement with lactated Ringer's solution and blood (3:1) until the MAP reach 80mmHg, following that a laparotomy was performed, followed by the Pringle's maneuver for 15 minutes and then total liver reperfusion lasting 60 minutes.

Surgical procedure – the animals were placed in a 12 hour fasting in the previous night. The surgical procedure were made under intraperitoneal anesthesia (IP) with a solution of cetamine cloridrate (80mg/kg – Cristalia, SP) plus xilazine cloridrate (10mg/kg – Bayer, SP) and atropine (0,05mg/kg – Halex Star, SP). After the anesthesia was induced they were placed in a supine position on a board with heat electrically controlled.

With the precautions against contamination (asepsis technique), the right carotid artery and right jugular vein were dissected, and a polyethylene catheter (PE 40), previously heparinezed, was place on each vessel. The artery was used to perform the bleeding and so establish a hemorrhagic shock, and to take blood sample. The vein used for volemic replacement. At the same time, the right femoral artery was exposed and a polyethylene catheter (PE 40), previously heparinazed, placed on it for control of the MAP. The following parameters were performed continuously: electrocardiogram (ECG), MAP, heart rate (HR), respiratory rate (RR); rectal temperature (kept between 36°C - 38°C by external heating); electrolytes (Na^+, K^+); base excess (BE); serum lactate and pH, all via arterial samples.

The controlled hemorrhagic shock with low tissue perfusion was monitored through pH, base excess (BE), serum lactate measurements. This same shock was attained through bleeding at regular intervals comprising 5% of the volemia each time, until the MAP reached 40mmHg for the Control and the Total groups. Further bleeding was performed if indicated. A laparotomy paying due care to avoid excessive bleeding was made. The hepatoduodenal ligament was identified and clamped (Pringle's maneuver) with soft arterial clamps. After the repefusion period of 60 minutes, the animals were killed.

Tissue processing – The brain were removed, cleaned with saline solution, and fixed in formal aldheid at 10% and embedded in paraffin. The right frontal lobe sections were cut (4 mm) and then, stained with hematoxilin/eosin. Counting of neutrophils per field in the cortical layer of the brain was done. A random study of this material (laminae) was undertaken, and the arithmetic mean of the neutrophils count in 10 microscope fields (magnified 400X) was recorded.

Statistic analysis – For comparison among the

groups the ANOVA method (Variance Analysis) was used. If a difference was noted, further study using the Student-Neuman-Kuels method was made. A significant level of 5% ($p < 0.05$) was used.

Results

Hemodynamic parameters – for each group, and through out all the phases of the present study, MAP, HR and RR were used to evaluate the hemodynamic status of the animals. Statistical analysis were carried out during the following periods: Initial Vital Parameters (IVP) – beginning of the reperfusion period (RP0) – 30 minutes thereafter (RP30) – after 60 minutes (RP60) for all groups during the experiment. Figure 1 shows details of MAP for the different groups.

Comparing the MAP at the start of the experiment (IVP), no significant differences were found among the groups ($p=0.265$); in the initial period of reperfusion (RP0) the MAP was different for the groups ($p<0.0001$) – the exceptions were the groups Pringle and Total ($p=0.1230$) with values ranging from 43.25±3.0 for the Pringle group and 46.00±14.6mmHg for the Total group, respectively. At RP30 only the Control group showed significant difference from the others groups (MAP 96.5±14.6mmHg). At RP60 again the Control group showed a difference with the MAP above the others groups.

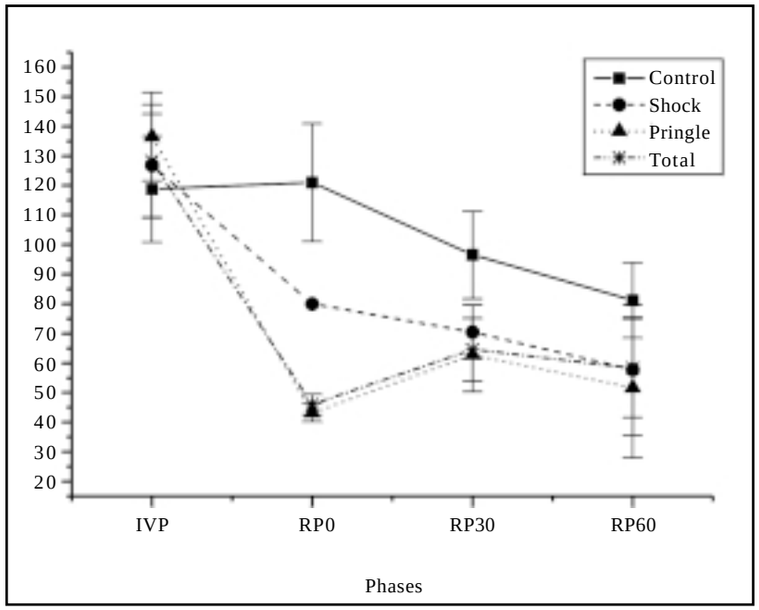

FIGURE 1 – Values of MAP on each experimental phase according with each group

Neutrophils accumulation – The amount of neutrophils per field in the brain, for all groups, after 60 min. of reperfusion is displayed in Table 1.

TABLE 1 – Neutrophils per field in the brain, according with each group.

Group Neutrophils / field	Control	Shock	Pringle	Total
Mean	0.12	0.12	0.02	0.14
SD	0.11	0.13	0.04	0.16
N	8	8	8	8

ANOVA: p = 0,196

Discussion

The use of the experimental models are helping to get better understanding of the I/R syndrome, especially in the molecular level inside the tissues. Studies on humans and in animal models show that inflammation can have a deleterious effect on the outcome of acute brain injury, such as stroke and head trauma[5,6].

On the other hand, until recently, the central nervous system (CNS) of mammals has been considered to be an immunologically privileged site because of the lack of lymphatic drainage, and its separation from the blood by the blood-brain barrier[7,8]. The BBB is comprised of the endothelial cells that line the capillaries of the brain, and plays a important role in controlling the access of inflammatory cells, such as circulating neutrophils or lymphocytes, and macromolecules into the brain tissue by virtue of its selective permeability. This restriction arises by the presence of tight intercellular junctions (zonulae occludens) between adjacent endothelial cells, a complex glycocalix, relative paucity of pinocytotic vesicles within endothelium of cerebral arterioles, capillaries, venules, absence of fenestra, and wrapping by astrocytes that constitute part of this barrier. Thus, this cells and structures in the local environment are required to maintain normal BBB function and these properties allow for the selective exchange of substances between the systemic circulation and the extracellular fluid compartment of the brain[9,10].

However, during inflammation in the CNS, circulating leukocytes readily gain access to brain parenchyma, and the molecular mechanisms involved in circulating cells recruitment across the BBB have been mostly studied in experimental autoimmune encephalomyelitis, the prototype model for human inflammatory demyelinating diseases of the CNS such as multiple sclerosis[8,11,12]. When inflammation is present, the BBB itself plays an important role in the inflammatory response by either producing or expressing a variety of cytokines, adhesion molecules, serine proteases, metalloproteinases, products of arachidonic acid metabolism, and nitric oxide[10,13,14,15]. Understanding the integral role of the BBB during inflammation is essential when creating and employing a therapeutic regime for the CSN diseases.

Pathological states (i.e. stroke, cardiac arrest, hemorrhagic shock state) can lead to reduced blood flow to the brain, potentially altering BBB permeability, and regulatory transport functions. Blood-brain barrier disruption leads to increased cerebrovascular permeability, an important factor in the development of ischemic brain injury and edema formation, which occur in the head trauma injury[16]. This suggest that although autoregulation maintains the cerebral blood flow, BBB transport mechanism are significantly compromised in states of reduced flow, and these alterations have a significant impact on brain homeostasis in pathological states, as verified in prolonged hemorrhagic shock state following important hepatic injury secondary to blunt abdominal trauma. However, the contribution of BBB opening to traumatic brain edema is not known[16,17]. Thus, many type of stimuli can alter the permeability characteristics of the BBB. Acute increased in arterial blood pressure beyond the autoregulatory capacity of cerebral blood vessels, volume replacement with hyperosmolar solution following hemorrhagic shock secondary to trauma injury, application of various inflammatory mediators known to be elevated during brain injury, and activation of blood-borne elements such as leukocytes can produces changes in permeability of the BBB[9,18].

On the other hand, death from abdominal trauma is most commonly due to liver injury, and with the advances made in rescue and early resuscitation, more patients are surviving to reach the operating room. However, the severely traumatized frequently requires temporary vascular occlusion to prevent exsanguination during surgical repair, a risk factor of postoperative morbidity and mortality in hepatic resection following trauma[19]. Often referred to as the Pringle's maneuver, the portal triad occlusion (PTO) is the standard method for reducing the risk of massive hemorrhage from the liver parenchyma while the liver injury is surgically repaired. This maneuver produces dramatic alterations in systemic hemodynamics during and immediately following PTO, but the exact etiology of these alterations remains unclear, and has been linked to ischemia of the liver as well as alterations of the splanchnic venous drainage resulting in intestinal ischemia, especially when duration of ischemia increases[20].

Furthermore, the liver has the highest fixed macrophages population (Kupffer cells) and following PTO, the release of free radical, endotoxin, tumor necrosis factor-a (TNF-a), and activation of Kupffer cells and neutrophils may lead to capillary endothelial injury, increased capillary permeability, and loss of plasma volume. The end result is progressive hypovolemic shock and cardiovascular and metabolic collapse[19,20]. Consequently, in these patients, the volemic compromise and tissue ischemia, if followed by a volemic replacement (reperfusion) treatment, becomes more important in the development of organic lesions and, depending of the affected organ and from the time it takes from the beginning of stoppage of the blood

flow, it may cause lesions in distant organs due to excessive production of oxygen radicals, highly reactive and cytotoxic[4,5]. In this sense, some studies have shown that, in the initial phase of the hemorrhagic shock, the neutrophils play an important role in the establishment of the ischemia and reperfusion syndrome, because they adhere (and get activated) to the endothelial cells, freeing the superoxide radicals and the proteolitic enzymes that produces the tissue lesions. These are the most important components responsible for the inflammatory process[21].

Otherwise, the response of individual organs to ischemia varies depending on the autoregulatory capacity of an organ in hypovolemic state, its energy stores, basal metabolic demands and ongoing functional activity. The brain is an organ with high energy demands, poor ischemic tolerance and its autoregulatory capacity is, however, also efficient in low-flow states. Critical metabolic alterations therefore do not occur in the brain until late shock when tissue perfusion becomes severely compromised[9,16]. On the other manner, the metabolism of the liver is critically disturbed in shock state. The energy reserve of this organ, expressed as the energy charge or phosphorylation potential, is rather poor in comparison to metabolic requirements of their parenchymal cells, therefore, becomes disturbed early in the course of hemorrhagic hypotension, and failure of liver function is frequently seen in shock states[19,20].

In peripheral tissues, neutrophils are the first circulating leukocytes to arrive at the site of injury. When they accumulate in blood vessels, neutrophils may bring about a reduction in blood flow and occlusion of microvessels. Although mechanisms appear to have evolved to restrict neutrophil entry to the brain parenchyma, it is clear that neutrophil recruitment is a feature of acute brain injury following stroke or trauma. Experimental studies using the permanent, or transient, focal ischemia model in rats have shown that neutrophil depletion decreases cerebral tissue damage, and in models of traumatic brain injury there is also a correlation between neutrophil accumulation and the development of cerebral edema[16,17]. These studies highlight that factors, such as BBB, are responsible for restricting neutrophil recruitment to the brain, and when the brain is compromised after severe injury, cytokines become important mediators of neutrophil recruitment. Recently, Bernardes-Silva[18] and colleagues have observed that 85% of the neutrophil recruitment induced by a proinflammatory cytokine is P-selectin-dependent but, surprisingly, E-selectin blockade had no effect on neutrophil recruitment to the brain parenchyma. Tsao *et al*.[8] has described that the increase in BBB permeability depended primarily on the action of TNF-a on microvascular vessels. They observed that the TNF-a produced in the bloodstream by leukocytes during sepsis induced the increase in BBB permeability suggesting the possibility that the peripheral bacteria and neutrophils may gain an entry to invade the brain through the BBB opening induced by TNF-a.

Moreover, it is often stated that the presence of the BBB poses an impenetrable obstacle to leukocytes attempting to exit the vasculature, and it is assumed that when the BBB is damaged, leukocyte recruitment is enable[18]. The hemorrhagic shock state associated, or not, with hepatic ischemia (Pringle's maneuver) can lead to stimulate the production and release of pro-inflammatory mediators such as cytokines and prostaglandins by leukocytes, endothelial cells, astrocytes, microglial cells and other cells in the CNS, which lead to an increase in the permeability of the BBB. This would trigger transendothelial migration of neutrophils and leakage of plasma proteins that could further damage the brain[19,22]. However, recent evidence in animals models shows that this is not the case[23]. Anthony *et al*.[24] have demonstrated that although the injection of inteleukin-1b (IL-1b) into the brain parenchyma of adult rats does not induce the recruitment of leukocytes at the site of injection, they are present in the meninges and the choroid plexus.

Conclusion

There was not statistical difference in the accumulation of neutrophils in the brain among the different groups studied. This suggest that in the I/R syndrome, associated or not with hepatic ischemia, in spite the fact, that the neutrophils accumulate in the micro vascular endothelium of the choroid plexus, they are not able to cross the blood-brain barrier toward the inner portion of the cerebral parenchyma.

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Acknowledgment

We would like to thanks to Mr. Edilson Silva Junior for helping on the translation of the present paper.

Mantovani M, Fontelles MJ, Hirano ES, Morandin RC, Schenka AA. Recrutamento de neutrófilos através da barreira hematoencefálica: importância da isquemia hepática pós-traumática. Acta Cir Bras [serial online] 2003 Set-Out;18(5). Disponível em URL: http://www.scielo.br/acb

RESUMO – Objetivo: estudar o efeito da isquemia e reperfusão hepática total sobre acúmulo de neutrófilos no cérebro de ratos, em condições de normalidade e submetidos ao estado de choque hemorrágico controlado. **Métodos:** Foram utilizados 32 ratos Wistar, machos, distribuídos em quatro grupos de oito animais cada: Grupo Controle, submetido aos procedimentos padrões com um período de 60 minutos de observação; Grupo Choque, submetido a choque hemorrágico controlado (PAM=40mmHg, 20 min) seguido de reposição volêmica (Ringer lactato + sangue, 3:1) e reperfusão (60 min); Grupo Pringle, submetido à isquemia hepática total (15 min) e reperfusão (60 min); Grupo Total submetido a choque hemorrágico controlado (15 min) seguido de reposição volêmica (Ringer lactato + sangue, 3:1) mais isquemia hepática total (15 min) e reperfusão (60 min). A dosagem do lactato arterial e déficit de base foram utilizados para caracterizar o estado de choque hemorrágico com baixa perfusão tecidual. Após a eutanásia dos animais, procedeu-se à contagem de neutrófilos no cérebro. **Resultados:** a contagem de neutrófilos mostrou que não houve diferença estatística entre os grupos (p=0.196). Grupo Controle 0.12±0.11, Choque 0.12±0.13, Pringle 0.02±0.04 e Total 0.14±0.16. **Conclusão:** Em ratos submetidos a estado de choque hemorrágico controlado associado à isquemia hepática total de 15 minutos, seguido de 60 minutos de reperfusão, não ocorreu acúmulo significativo de neutrófilos no cérebro.

DESCRITORES – Choque hemorrágico. Manobra de Pringle. Cérebro. Neutrófilos.

Conflict of interest: none
Financial source: none

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Data do recebimento: 15/07/2003
Data da revisão: 28/07/2003
Data da aprovação: 05/08/2003