Article

Arq Neuropsiquiatr 2010;68(6):918-922

Effect of N-acetylcysteine on vasospasm in subarachnoid hemorrhage

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ABSTRACT

Vasospasm remains an extremely serious complication that affects patients presenting with subarachnoid hemorrhage (SAH) due to ruptured intracranial aneurysms. The current therapeutic armamentarium is still insufficient in many cases, and the search for new therapies is necessary. In this study, we evaluated the effect of N-acetylcysteine (NAC) on cerebral arterial vasospasm using an experimental model. Twenty-four wistar rats were divided into 4 groups: [1] Control, [2] SAH, [3] SAH+NAC and [4] SAH+Placebo. The experimental model employed double subarachnoid injections of autologous blood. The proposed dose of NAC was 250 mg/kg intraperitoneally per day. We analyzed the inner area of the basilar artery to assess the action of NAC. The experimental model proved to be very adequate, with a mortality rate of 4%. The inner area of the basilar artery in the SAH group showed significant difference to the control group (p=0.009). The use of NAC significantly reduced vasospasm as compared to the untreated group (p=0.048) and established no significant difference to the control group (p=0.098). There was no significant improvement with the administration of placebo (p=0.97). The model of the dual hemorrhage proved to be very useful for vasospasm simulation, with overall low mortality. The administration of NAC significantly reduced vasospasm resulting from SAH, and may represent a new therapeutic alternative.

Key words: subarachnoid hemorrhage, vasospasm, N-acetylcysteine.

Efeito da N-acetilcisteína sobre o vasoespasmo na hemorragia subaracnóidea

RESUMO

O vasoespasmo arterial encefálico continua sendo uma complicação extremamente grave que acomete pacientes com hemorragia subaracnóidea (HSA) por ruptura de aneurismas. O arsenal terapêutico atual ainda, em muitos casos, é insuficiente e a busca de novas alternativas terapêuticas torna-se necessária. Neste estudo, avaliamos a ação da N-acetilcisteína (NAC) sobre o vasoespasmo arterial encefálico em um modelo experimental. Foram utilizados 24 ratos wistar divididos em 4 grupos: [1] Controle, [2] HSA, [3] HSA+NAC e [4] HSA+Placebo. O modelo experimental utilizado foi o da dupla injeção subaracnóidea de sangue autólogo. A dose proposta da NAC foi de 250 mg/kg/dia por via intraperitoneal. Foi analisada a área interna da artéria basilar para avaliação da ação da NAC. O modelo experimental mostrou-se excelente com mortalidade de 4%. A mensuração da área interna da artéria basilar do grupo HSA demonstrou diminuição significativa em relação ao grupo controle (p=0,009). A administração da NAC reduziu significativamente

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Received 3 March 2010
Received in final form 29 June 2010
Accepted 6 July 2010

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o vasoespasmo em relação ao grupo não tratado (p=0,048) e estabeleceu diferença não significativa em relação ao grupo controle (p=0,098). Não houve melhora significativa com administração de placebo (P=0,97). O modelo da dupla hemorragia mostrou-se bastante útil para reprodução do vasoespasmo, com baixos índices de mortalidade. A administração da NAC diminuiu significativamente o vasoespasmo decorrente da HSA, podendo representar uma nova alternativa terapêutica.

Palavras-chave: hemorragia subaracnóidea, vasoespasmo, N-acetilcisteína.

Subarachnoid hemorrhage (SAH) is one of the most frequent intracranial hemorrhages, representing about 1 to 7% of all strokes recorded[1]. Excluding head injury, which is the most common cause, the rupture of cerebral aneurysms represents approximately 75 to 80% of the events leading to SAH[2].

SAH due to a ruptured cerebral aneurysm is an acute and extremely severe event, with very high mortality and morbidity rates, so that a prompt diagnosis and treatment are essential to the preservation of life and to lessen complications. The natural history of disease shows that about 35% of patients who survive the initial SAH develop symptomatic cerebral arterial spasm leading to secondary ischemia (delayed ischemic neurological deficits), and the morbidity and mortality rates estimated for this complication at about 56% of cases[3].

Several theories on the pathogenesis of vasospasm have been explored in recent years, mostly supported by findings derived from basic research experiments involving animals. Potential treatments for vasospasm have been proposed according to these theories, but mostly with favorable partial response, reinforcing the hypothesis that vasospasm has a multifactorial origin. The main mechanisms theoretically involved include immune response, vascular proliferation, inflammatory response of the arterial wall, excess of endothelins, neurogenic factors and recently the depletion of nitric oxide and the oxidative stress caused by excessive production of free radicals[4]. The oxidative stress characterized by lipid peroxidation and increased levels of reactive oxygen species and other free radicals has been linked to the etiology of vasospasm after subarachnoid hemorrhage.

N-acetylcysteine (NAC) is a compound known for its potent antioxidant action, acting directly as a free radicals scavenger and as a precursor to other compounds and antioxidants in the body, including the nitric oxide (NO)[5]. Currently it is used in humans to treat various diseases, with application in the respiratory, digestive and immune systems and also tested in experimental models for the treatment of many other diseases[6-10].

Although current extensive research on the use of NAC for the treatment of neurological disorders, its use and potential benefits in the SAH remain poorly studied and still need to be further elucidated. However, recently, LU et al., demonstrated that NAC is able to suppress oxidative stress in the brain of rats with SAH[11].

Given these assertions, the application of antioxidants such as NAC in combating the genesis and maintenance of vasospasm in SAH appears to be a promising therapy. The aim of our study was to evaluate the action of NAC on vasospasm in an experimental model of SAH in rats.

METHOD

Experimental model

Model of "double subarachnoid hemorrhage": Technique: The animals were anesthetized with an intraperitoneal injection of ketamine 50 mg/ml - 90mg/Kg + Xylazine 2% - 10 mg/Kg. Ceftriaxone (100 mg/kg intramusculary) was used for antibiotic prophylaxis to each injection of blood. The animals were positioned on the operating table which was adapted for head fixation. Occipito-cervical trichotomy, local antisepsis and disinfection with alcoholic iodine solution, placement of a fenestrated surgical prep and incision of occipito-cervical skin exposing the atlanto-occipital membrane were performed. The cisterna magna was punctured through the atlanto-occipital membrane with a Butterfly™ 25. About 0.1 to 0.15 ml of CSF was removed and 0.3 ml of fresh blood, collected at the same time from the femoral artery by direct arterial puncture, was injected slowly. After the injection of blood, the Butterfly™ was removed and a suture of the skin with mononylon 4.0 was performed. The same procedure was repeated about 48 hours after. Sacrifice occurred seven days after the first bleeding by transcardiac perfusion of 10% buffered formalin. The animals were anesthetized again using the same anesthetic protocol and positioned with the exposed thoracic region. Thoracotomy followed by catheterization of the left ventricle for the infusion of 10% buffered formalin with a constant pressure of 20 cm H_2O and puncture of the right atrium for blood removal were performed. After the infusion, the animals were repositioned with occipital region exposure. Opening and expansion of the occipito-cervical incision made prior to injection of blood were performed, followed by craniotomy with kerrison rongeur, with opening of the dura-mater and microsurgical dissection with a DF Vasconcelos MC-1187 surgical microscope. After sacrifice, brains were removed, immediately

placed in 10% buffered formalin and sent to be included in paraffin. Cuts were made with microtome to the fullest extent of the basilar artery.

Groups

This study utilized 24 male Wistar rats with average weight of 350g divided into 4 groups with n=6: [1] Control (Sham operated), [2] SAH, [3] SAH + NAC and [4] SAH + Placebo.

Administration of NAC

The NAC (Fluimucil™ Injectable 100 mg/ml - lot 08E03/01 - Zambon) was administered intraperitoneally at a dose of 250 mg/kg/day from the time of first bleeding. The same volume of 0.9% saline was administered in the placebo group. The SAH and control groups received no treatment at all.

Preparation of slides and measurements.

Five slides were randomly selected from the middle third of the basilar artery for direct microscopic analysis of the internal area. (Olympus BX40 / JVC TK1280E). Images were acquired and analyzed by Image-Pro Plus® (version 6.3.0.512 for Windows - Media Cybernetics Inc.) The analysis of images was performed by a professional who was not acquainted of the groups division.

Statistical analysis

Statistical analysis was performed using Student's t test and ANOVA. Differences were considered significant if $p<0.05$. Values were expressed as the mean±standard deviation.

Ethical

All procedures were performed in accordance to the rules for animal use in research. The study was approved by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre under protocol number 854/09.

RESULTS

SAH and inner area reduction of the basilar artery were observed in all animals subjected to the experimental model. Only 1 of 23 animals subjected to double injection of blood died before the end of the experiment (Figure and Graphic 1A).

The inner area of the basilar artery obtained by analysis of the slides was 1.481 mm^2 (±0.337) in the Control group, 0.797 mm^2 (±0.200) in the SAH group, 1.146 mm^2 (±0.225) in the SAH+NAC group + 0.803 mm^2 (±0.249) group SAH + Placebo group (Graphics 1B and 2).

DISCUSSION

The arterial cerebral vasospasm present in SAH continues to represent an important complication, adding difficulty to the management of patients suffering from ruptured brain aneurysms. A better understanding of pathophysiological mechanisms has allowed the proposal of new treatments.

The association of oxidative stress with SAH and cerebral vasospasm has been widely studied[12-18].

Brain tissue is more prone to oxidative damage in relation to other organs due to its high concentration and consumption of oxygen[19]. It is known that the extravasated blood into the subarachnoid space allows the release

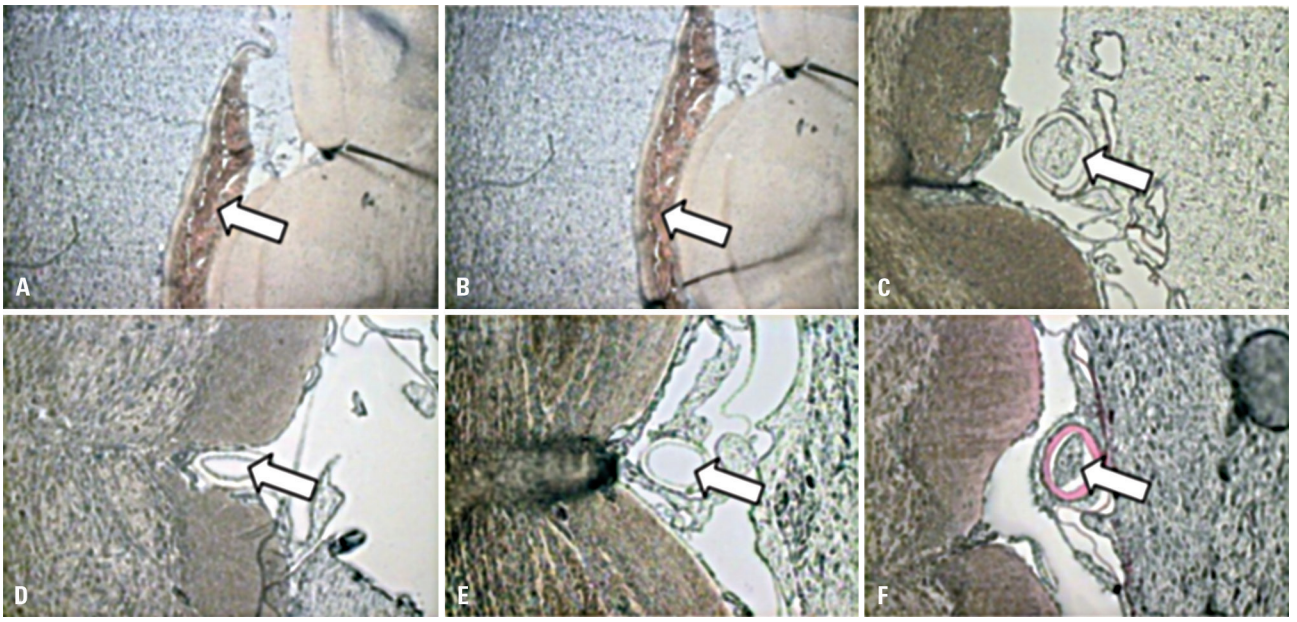
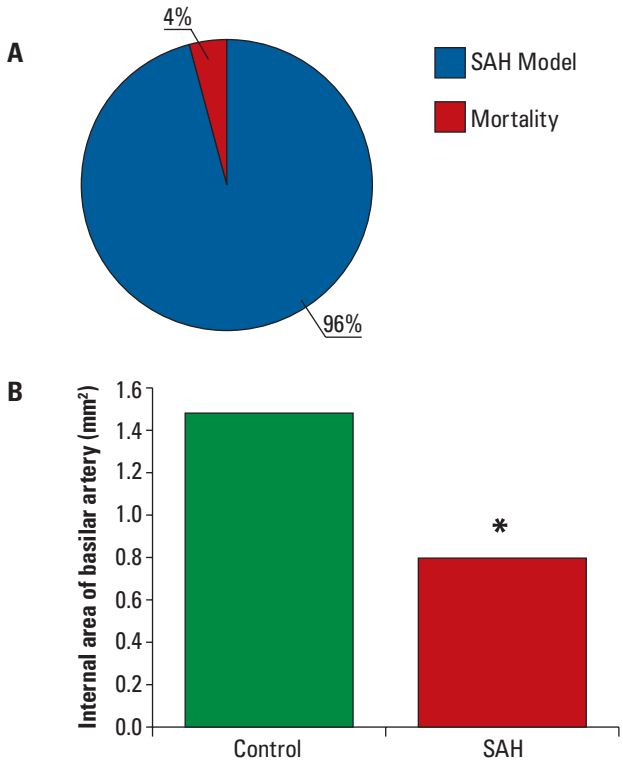
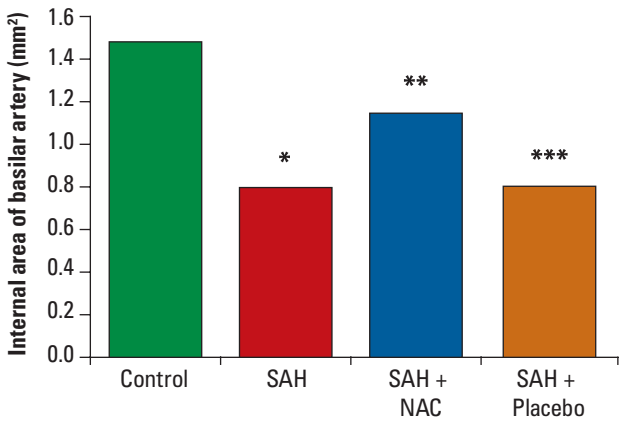


Figure. [A and B] Brainstem with subarachnoid hemorrhage (SAH). [C] Section of the basilar artery - control group. [D] Section of the basilar artery - SAH group. [E] Section of the basilar artery - SAH + NAC group. [F] Section of the basilar artery - SAH + placebo group. 10 × lens.



Graphic 1. [A] Mortality rate of experimental model. [B] Experimental model. Values of the internal area of basilar artery. Control and subarachnoid hemorrhage (SAH) groups. Values expressed as the mean±standard deviation. *p<0.01.



Groups	p
* Control × SAH	0.0094
SAH + NAC × SAH	0.0481
** SAH + NAC × Control	0.0983
SAH + NAC × SAH + Placebo	0.0905
*** Control × SAH + Placebo	0.0168
SAH × SAH + Placebo	0.9743

Graphic 2. Values of the internal area of basilar artery. Control, subarachnoid hemorrhage (SAH), SAH + N-acetylcysteine (NAC) and SAH + Placebo groups. Values expressed as the mean±standard deviation.

of its constituents, including free hemoglobin (Hb). Hb is a protein that contains iron, which acts directly as a free radical and also participates in reactions that favor the production of other free radicals[20].

Free radicals have an important role in the pathogenesis of SAH and cause oxidative damage to lipids and proteins of cell membranes. Increased levels of free radicals can cause damage to virtually all cellular components, including DNA, lipids and proteins, leading to damage of neurons, glial cells and blood vessels.

In cases involving the production of free radicals, these are countered by the body's natural antioxidants, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). SOD represents the first line of defense against oxidative stress, catalyzing the dismutation of superoxide anions into hydrogen peroxide. Therefore, hydrogen peroxide is converted into water and molecular oxygen by GSH-Px metabolism[21].

As aggression is perpetuated, the levels of endogenous antioxidants decrease, favouring the appearance of damage caused by oxidative stress.

Lipid peroxidation is a major consequence of the injury mediated by free radicals. Peroxidation of membrane phospholipids consists of a chain reaction that can continue until the substrate is completely consumed, and a potential cause of structural and functional damages making difficult or impossible membrane transports[22].

The relation between the depletion of NO production in SAH with cerebral vasospasm is also a remarkable fact in the pathogenesis. Nitric oxide acts as an important arterial vasodilator. Clatterbuck et al. obtained favorable results against vasospasm after reposition of nitric oxide[12,23,24].

In this study, we aimed to evaluate the action of NAC in an experimental model of SAH and cerebral vasospasm in rats. The NAC is a compound with potent antioxidant properties and ability to increase levels of endogenous antioxidants and NO.

Several experimental models can be used to study vasospasm in SAH. Each of them has advantages and problems. It's known that primates and dogs represent the best choices for overall correlation with human vasospasm, but the costs are very high and they are not easily available. Rats represent a choice for initial screening studies for pharmacologic prevention and reversal of vasospasm, but we know that these models have a fair overall correlation with human vasospasm[4]. The experimental model used, double hemorrhage of autologous blood in rats, proved to be adequate to our study, and only one of the twenty-three animals that underwent double in-

tracisternal blood injection died before the end of the experiment, establishing a mortality rate of 4.34%. This is an experimental model of easy execution and low cost, supporting the adequacy for this type of study. The measurement of the internal area of the basilar artery on SAH group showed significant decrease to the control group (p=0.009), confirming the fidelity of the experimental model for the development of vasospasm. Eventual hemodynamic alterations due to the femoral puncture and arterial blood removal could represent a bias to the experimental model, but we believe that the amount of blood extracted from femoral artery is not capable to produce this type of alteration. Moreover, this procedure was performed for all groups, so if any alteration had happened, it would be common for all groups.

The administration of NAC significantly reduced vasospasm in relation to the untreated group (p=0.048) and established no significant difference to the control group (p=0.098). The beneficial effect of NAC is probably due to its ability to directly combat free radicals and also promote the production of NO and the body's natural antioxidants such as GSH-Px and SOD, as described by Lu et al.[11].

The results of this study strongly suggest that oxidative stress plays an important role in the development of vasospasm in SAH, confirming previous studies. The administration of NAC was capable to significantly reverse the vasospasm, even in a double hemorrhage model, which is known to cause significant arterial spasm.

The precise pathophysiological mechanisms that correlate oxidative stress present in the HSA with the emergence and maintenance of cerebral arterial vasospasm still remain poorly elucidated, but therapy with antioxidants may represent a new way for the best treatment of this serious disease.

In conclusion, the double hemorrhage model in rats has shown to be useful for vasospasm simulation. A significant difference of the inner area from the basilar artery between SAH and Control groups was observed, with low mortality rate.

There was no significant improvement with the administration of placebo.

The administration of NAC significantly reduced vasospasm resulting from SAH and established no significant difference to the control group.

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