Neuroendocrinology Letters No.5, October Vol.24, 2003
 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Free radicals after painful stimulation are influenced by antioxidants and analgesics

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Submitted: *September 15, 2003*
Accepted: *September 27, 2003*

Key words: **nociception; free radicals; antioxidants; rats**

*Neuroendocrinol Lett 2003; **24**(5):304–309 pii: NEL240503A01*

Abstract

OBJECTIVES: To study the balance between the pro-oxidative and antioxidative defence system after repeated painful stimulation in rats and the efficacy of the administration of different antioxidants (vitamins C, E, A, and selenium), analgesics (acetylsalicylic acid, morphine), and their combinations.

METHODS: Mechanical clamping of both hind limbs was applied for 10 min for 5 consecutive days in adult male Wistar rats. The tail-flick latency was measured before and after a 5-day nociceptive stimulation with or without the substance application. The reactive oxygen species (ROS) were determined in the sensorimotor cortex.

RESULTS: Painful stimulation increased lipoperoxidation which persisted for up to 15 days after it had been discontinued. A simultaneous injection of antioxidants decreased the levels of TBARS, SOD and GSHPx; however, antioxidants applied one week prior to the painful stimulation were ineffective. A simultaneous injection of analgesics reduced stress-induced analgesia caused by the nociceptive stimulation, but did not affect lipoperoxidation.

CONCLUSIONS: A combination of antioxidants with analgesics normalized both the oxidative stress and functional (the tail-flick latency) indicators. These results suggest that the administration of antioxidants in pain treatment may be employed to decrease the doses of analgesics and to prevent the negative impact of reactive oxygen species on nociception.

Introduction

The entire evolution of living organisms is characterized by the mutual rivalry of free radicals and antioxidants. Both free radicals and antioxidants have developed and they have always influenced the viability of living organisms. Many diseases and pathological syndromes are caused by oxidative stress, i.e. by an imbalance in favor of free radicals. The most frequent consequences of oxidative stress are atherosclerosis, diabetes mellitus, tumors, cataract and ageing. The effect of free radicals, as one of the oldest chemical stimuli, on the phylogenetically youngest and the best-organized biological structure – the central nervous system (CNS) – has been relatively seldom studied. Up to now, reactive oxygen species (ROS) have been measured in Alzheimer's [1] and Parkinson's [2] diseases, epilepsy [3], trauma and amyotrophical lateral sclerosis. However, information about the influence of free radicals and antioxidants in the CNS during pain is almost completely lacking.

ROS are produced in the brain during cellular respiration and their rate is accelerated during brain insults [4]. Oxidative stress is the result of an imbalance between higher free radical production and a lower degradation. Astrocytes with a high intracellular concentration of antioxidants are more resistant to free radicals than oligodendrocytes and neurons [5]. ROS which cause vasospasms in the brain [6] are either produced during reperfusion after prior ischemia and are very likely associated with pain. According to the chemical composition, the brain cortex with its high content of neurons is much more sensitive to ROS damage than glial cells. We were therefore interested, if ROS were produced in the brain tissue during painful stimulation.

It is possible to directly detect free radicals using electron paramagnetic resonance (EPR) or assess some substances or antioxidative enzymes indirectly, which would indicate oxidative stress during ROS generation. A rise of the superoxide content enhances the superoxide dismutase (SOD) activity. During this reaction, hydrogen peroxide is produced. This substance permeates all tissues and membranes and exerts a much longer action than the superoxide or free hydroxyl radicals, which are produced by the Fenton reaction from hydrogen peroxide during catalytic action of Fe^{2+}, or Cu, Cd, Ni and others. The degradation of peroxides in the brain is caused mainly by glutathione peroxidase (GSHPx). Lipoperoxidation is another criterion of oxidative stress. It is detected by malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS). Furthermore, other substances such as lipid hydroperoxides, lipofuscin-like pigments, isoprostanes etc. may also serve as markers of lipoperoxidation. The antioxidative capacity (AOC) then indicates the general ability of the homogenate to detoxicate ROS.

It has been known for the long time that lipoperoxidation in the brain can damage nervous structures and their functions by decreasing Na/K ATP-ase activity. It decreases membrane fluidity and modifies lipids into lipid hydroperoxides. These can damage cell membranes and facilitates the permeability of ions, especially of Ca^{2+}, through membranes [7]. The rat brain cortex is effectively protected against lipoperoxidation and free radicals. Acute stress activates the antioxidative system, which inhibits lipoperoxidation in the brain, contrary to other organs, where lipoperoxidation takes place more intensively [8]. In our work, we studied the mechanism by which pain influences the production of free radicals and how effective is the administration of different antioxidants and analgesics, or their combinations.

Material and Methods

Adult male rats of the Wistar strain were used for all the experiments. They were bred according to good laboratory practice principles (constant lighting, alternated 12 hours light and darkness, fed ad libitum, 8 animals per cage). Altogether 122 rats with body weight 170–200 g were used. In the first experiment we used 38 animals in three groups (12–13 animals in each group). In the second experiment 122 rats were used in ten groups (5 to 10 animals in each group). All animal procedures were in strict accordance with the Declaration of Helsinki and guidelines of the Ethics Committee of the International Association for The Study of Pain [9] and were approved by the Animal Care Committee of the 3rd Faculty of Medicine, Charles University, Prague. Suffering of the animals was reduced to the minimum.

Painful stimulation: Our own model of mechanical pain was used [10]. The mechanical clamping of both hind limbs was applied for 10 min for 5 consecutive days. The crocodile clamps were placed on the distal parts of hind limbs. The length of stimulation lasting for five days was estimated from our previous pilot studies, which showed that single or short lasting stimulation is ineffective to provoke the constant and stable biochemical changes [11]. The nerves of hind limbs were not damaged and the inflammation was not present. The neuropathologist did not find the angiogenesis and increased number of leukocytes in surrounding tissues (personal communication, M. Michal).

Biochemical determination: After the end of the 5th stimulation, the sensorimotor brain cortex was removed under the total anesthesia (Ketamine-Narkamon 90 mg/kg) and the local (Procaine 1%) anesthesia. The removed cortex was weighted (50–70 mg) and frozen immediately in liquid nitrogen. The tissue was homogenized after defreezing in homogenizer together with a 40 times grater volume of 0.01 mol/l phosphate buffer at pH 7.0 and then exposed to ultrasound for 10 seconds.

The SOD, GSHPx and AOC determination was performed by RANDOX sets (Crumlin, Northern Ireland) in an automatic analyzer Hitachi 717 (Boehringer-Manheim). MDA was assessed by TBARS method [12].

Antioxidants were injected intramuscularly in the following doses: Ascorbic acid (vitamin C) 3.5 mg/kg,

Trolox-α-tocopherol (water soluble vitamin E) 5 mg/kg, Axerophtol (vitamin A) 0.5 mg/kg and 0.28 ng/kg selenium in the form of $Na_2SeO_3.H_2O$.

Two kinds of analgesics were used: acetylsalicylic acid (Aspegic Synthelabo, 30 mg/kg i.m.) as a non-steroid anti-inflammatory drug representative and morphine (Morphine Biotica 1% 0.15mg/kg i.m.) as a representative of opioids.

Both antioxidants and analgesics were used in the doses derived from human recommended doses. Morphine was applied in the lower dose, which still provoked the clinical effect. The strong morphine analgesia induced by higher doses of morphine could mask the tested effect of antioxidants.

Tail flick. A tail-flick assay was performed using the Tail-flick analgesia meter (IITC Life Science USA Model 33). A beam of light was focused on the dorsal surface of the tail approximately 2.5 cm from the tail's tip. The intensity of the heat stimulus was adjusted so that the baseline latencies averaged between 3–4 s. The tail-flick latency was measured before and after the 5-day nociceptive stimulation. Each nociceptive test consisted of three measurements.

Statistical analysis

The data were analyzed by 2x4 two-way ANOVA with two factors of nociception (with and without the nociceptive stimulation) and four grouping factors (control, antioxidants, acetylsalicylic acid, morphine). Design for repeated measures was used because of two samples of sensorimotor cortex from both hemispheres were evaluated. *T-test* was utilized to compare the groups treated simultaneously with antioxidants and analgesics with the same groups treated only by one substance.

Results

In the first experiment, the values of TBARS and SOD were significantly enhanced after 5-day painful stimulation ($F(2,35) = 42.68$; $p < 0.0001$; $F(2,35) = 31.38$; $p < 0.0001$, respectively). The increase of GSHPx was not significant ($F(2,35) = 2.086$; $p = 0.1393$). The AOC values did not change ($F(2,35) = 0.676$; $p = 0.515$) (Fig.l). The increased values of parameters still persisted 15 days after the termination of painful stimulation, significantly in SOD only ($p < 0.0001$) (Fig .1).

In the second experiment the nociceptive stimulation influenced TBARS differently in different groups (interaction group x nociception $F(3,57 = 3.07$; $p = 0.0349$). TBARS are increased in saline, antioxidants and acetylsalicylic acid groups and decreased in morphine group. The combination of antioxidants with morphine decreased TBARS in the same manner as morphine itself ($p = 0.077$; $p = 0.0580$, respectively). The effect of the combination of acetylsalicylic acid with antioxidants had inverse effect ($p = 0.18$ and 0.024, respectively) (Fig 2).

The applied antioxidants significantly decreased the levels of SOD ($p = 0.0086$) and GSHPx ($p < 0.0001$) in control animals, probably due to replacement of the activity of endogenous antioxidants. The changes in stimulated rats were non-significant for SOD ($p = 0.3216$) but significant for GSHPx ($p = 0.013$) (Fig 2).

When antioxidants (vitamins A, C, E and selenium) were given simultaneously with analgesics, the SOD and GSHPx values decreased after the nociceptive stimulation if compared with the values after nociceptive stimulation in control animals (SOD: antioxidant and acetylsalicylic acid $p = 0.0538$; antioxidant and morphine $p < 0.0001$; GSHPx: antioxidant and acetylsalicylic acid $p = 0.0047$; antioxidant and morphine $p < 0.0001$). Morphine and acetylsalicylic acid exhibited similar effect as in the combination with antioxidants (Fig .2).

The levels of AOC were constant with the exception of acetylsalicylic acid group, which was decreased in comparison with the control group ($p = 0.0001$) but did not differ after the nociceptive stimulation ($p = 0.6519$) (Fig. 2).

The tail-flick latencies increased after the nociceptive stimulation as a result of stress–induced analgesia ($p < 0.0001$). The antioxidative mixture itself did not significantly influence the stress-induced analgesia ($p = 1$). Both acetylsalicylic acid and morphine in combination with antioxidants significantly reduced the

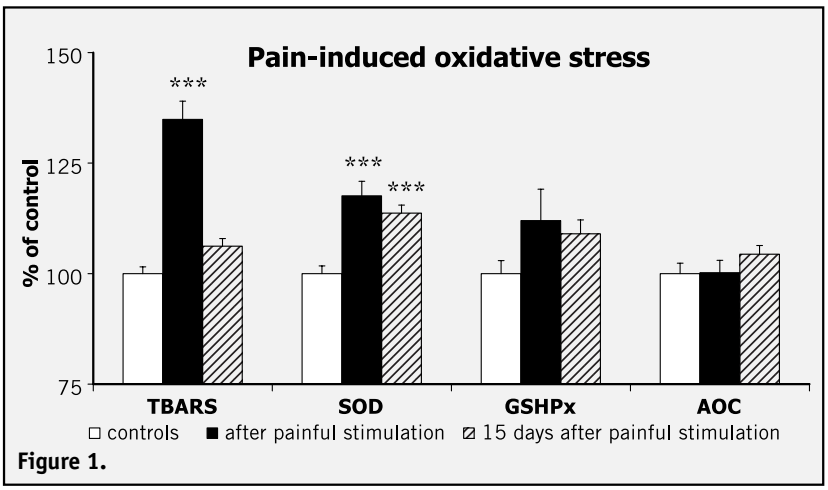

Figure 1.

Figure 1. Changes in thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and glutathion peroxidase (GSHPx) activity, and the antioxidative capacity (AOC) immediately after the termination of the 5-day painful stimulation (black columns) and 15 days (dashed columns) after the termination of the painful stimulation. The data are expressed as percentage (± SEM) of the control values in the first columns. Asterisks indicate significantly increased levels in comparison with the control values ($P < 0.001$).

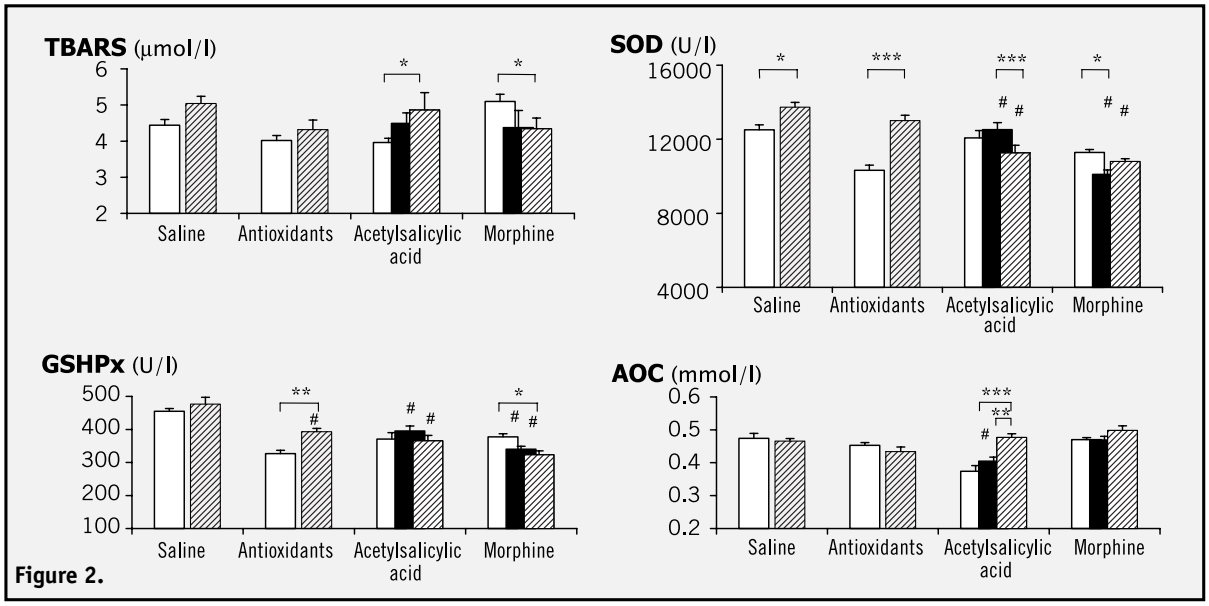

Figure 2. The effect of painful stimulation (hatched and black columns) on thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), glutathion peroxidase (GSHPx) and the antioxidative capacity (AOC) under saline (control) conditions and after the administration of antioxidants, acetylsalicylic acid and morphine. Black columns represent values after concomitant administration of antioxidants and analgesics immediately before the painful stimulation. White columns represent control values without painful stimulation in all groups. Asterisks indicate significant differences within the groups, the symbol # indicates significant differences between the stimulated rats from the control saline group and stimulated rats with the different types of treatment.
(*** P < 0.001, ** P < 0.01, * P < 0.05).

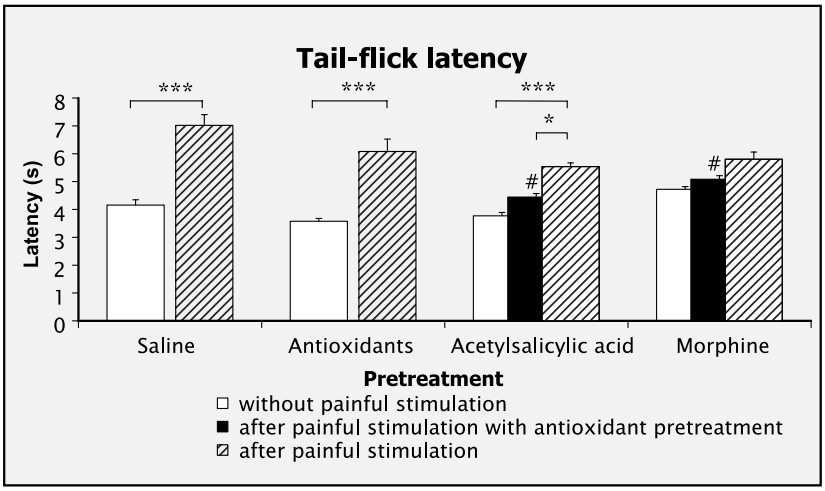

Figure 3. Changes in the tail-flick latency under control conditions (white columns) and after the 5-day painful stimulation with concomitant treatment with saline, antioxidants, acetylsalicylic acid, morphine (hatched columns), and combinations of antioxidants and analgesics (black columns). Asterisks indicate significant differences within the groups, the symbol # indicates significant differences between the stimulated rats from the control saline group and stimulated rats with the application of acetylsalicylic acid and morphine combined with antioxidants (black columns)
(*** P < 0.001, * P < 0.05).

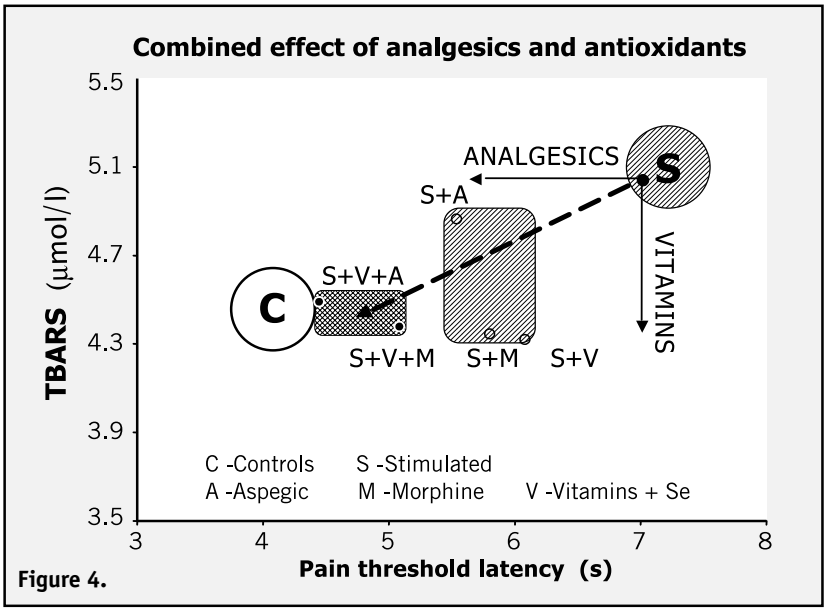

Figure 4. The relationship between TBARS as an indicator of oxidative stress intensity and the tail-flick latency as the marker of nociceptive stimulation stress. Symbol C shows the control values of non-stimulated animals, symbol S represents the relations after repeated painful stimulation where both TBARS and the pain thresholds were increased. The administration of vitamins during the painful stimulation (S+V) decreased TBARS but did not alter the pain threshold. On the other hand, analgesics (S+A, S+M) exerted a reverse effect since they decreased pain threshold by reducing the intensity of stress-induced analgesia but did not significantly affect the TBARS. The simultaneous administration of antioxidants and analgesics (S+V+A, S+V+M) changed both stress indicators by recovering their values approximately to control levels.

stress-induced analgesia ($p < 0.0001$ and $p = 0.0049$, respectively) (Fig . 3).

The Fig. 4 summarized schematically the obtained results. A simultaneous injection of the antioxidative cocktail and analgesics partially normalized both biochemical (TBARS, SOD, GSHPx) and functional (tail-flick) indicators of nociceptive stimulation. Behavioral data are more clearly expressed then the biochemical ones.

Discussion

Painful stimulation increases the production of free radicals and it increases lipoperoxidation (TBARS). The application of the vitamin mixture reduces lipoperoxidation thus reducing the TBARS values. Free radical superoxides stimulate SOD activity; the administration of the vitamin mixture probably attenuates superoxide production or scavenges the already produced superoxide. This may be the reason why SOD levels are lower after repeated application of the vitamin mixture if compared with the controls. As mentioned in the Results section, the normalization of the level of antioxidative enzymes is probably caused by the exogenous supplementation of antioxidants (Vitamins E, C, A and selenium). But it is necessary that the antioxidative mixture should be applied immediately before the nociceptive stimulation. In our pilot study we proved that the application of antioxidative mixture applied one week before the nociceptive stimulation has no protective effect.

The increase of peroxides is mediated by SOD action. They are degraded by GSHPx, thus enhancing the activity of this enzyme. The antioxidative capacity should be increased by antioxidant application but because it is partially consumed during the removal of free radicals, its value does not change significantly. It was found out that in chicken tissue the supplementation with antioxidant vitamins (E, C, A and trace elements Se, Cu) decreased lipid peroxide levels, while SOD and GSHPx activities increased in heart, liver, kidney and brain and CAT (catalase) decreased [13]. The application of antioxidants increases the antioxidative capacity and thus enhances the protection against the consequences of pain. Antioxidants, when properly used, may not only protect CNS against free radicals, but they are also able to decrease the sensation of pain. The protective role of morphine is dependent on an intracellular antioxidant system such as GSHPx [14].

The permeability of antioxidants through the blood-brain-barrier is of special importance [15]. It may be penetrated only by some of them (for example melatonin, some flavonoids, 21-aminosteroids and others). Pain itself can result in a higher permeability of antioxidants through the blood-brain barrier. Hence, their application during painful stimulation is more effective than a preventive one. It was demonstrated that stress (immobilization or forced swimming) could increase the permeability of the blood-brain barrier [16, 17]. During our procedure of eliciting pain, immobilization stress was also involved. The changes of MDA and antioxidants seem to be specific for the pain mechanisms because they are not influenced by the different types of stressors (immobilization, hypoxia) [18, 19]. Free radicals scavengers (DMSO-dimethylsulfoxide 50 % and NAC N-acetylcystein) are used for the treatment of CRPS (complex regional pain syndrome) and the pain during this disease [20].

In all animal models of acute pain and especially in mechanical and neuropathic pain, the restrain stress is always present. Each type of pain provokes the stress reactions and therefore it is very difficult to strictly differentiate between the pain and stress contributions in nociceptive responses. In patients with posttraumatic stress disorder the mean GSHPx, SOD, CAT activities and MDA levels did not differ from those of controls. However, in patients, the GSHPx and SOD activities were significantly positively correlated with symptoms severity [21]. In our study, the lower activities of GSHPx and SOD were found after the applications of antioxidants, analgesics and their combinations parallel with the decreased magnitude of stress-induced analgesia. These changes could reflect better coping with nociceptive stress. The increasing levels of MDA, SOD and GSHPx were found in patients with panic disorder [22].

The analgesic effect of morphine was probably potentiated by endogenous opioid system, which was activated by immobilization stress. We supposed that the stress-induced analgesia was of the opioid type. In the case of non-opioid analgesia the effect of morphine could be less intensive [23]. It was also proved that morphine has antioxidative effect. Childs *et al.* proved that water-soluble antioxidants (Vitamin C and N-acetylcysteine (NAC)) could act transiently as pro-oxidants in humans during inflammatory conditions [24]. Similar results referred also Khalil and Khodr that the early intervention with antioxidants could exert a negative effect on repair of injured nerves [25]. On contrary the antioxidants NAC significantly reduced the soft tissue damage and shortened the repair period [26].

It is known that idiosyncratic NSAID drug also induces oxidative stress [27]. The changes of the final biochemical products of free radicals effect during nociceptive processes were also proved by other authors. Chi *et al.* demonstrated in patients with acute abdominal pain the increasing level of MDA and decreasing level of total antioxidant capacity [28]. Also glutathione may protect oxidative stress and together with NAC could serve as an effective pre-emptive therapeutic strategy in painful nerve injury [29]. Gazda *et al.* found the increasing superoxide production in sciatic inflammatory pain [30]. This is in agreement with our results as well as with the finding of de Bono that SOD-mimics minimizes pain [31].

From our results we can conclude that the administration of vitamins during the painful stimulation decreased TBARS but did not alter the pain threshold. On the other hand, analgesics exerted a reverse effect since they decreased pain threshold by reducing the intensity of stress-induced analgesia but did not significantly affect the TBARS. The simultaneous administra-

tion of antioxidants and analgesics changed both stress indicators by recovering their values approximately to control levels.

Acknowledgements

This study was supported by the grant IGA MZCR 4665-3, Research goal J13/98:111200005 and CNS LN00B122 MŠMT CR.

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