The evaluation of nociceptive intensity by using free radicals direct measurement by EPR method in the tail of anaesthetized rats

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Submitted: 2008-09-15 Accepted: 2008-10-26 Published online: 2008-12-29

Key words: **EPR – direct measurement in living; rats; nociceptive stimulation; hydroxyl and nitroxide free radicals; singlet oxygen; antioxidants**

Neuroendocrinol Lett 2008; **29**(6):1007–1014 **PMID:** 19112388 NEL290608A17

Abstract

OBJECTIVES: The aim of the study was to demonstrate the ability to measure free radicals and singlet oxygen, using EPR methods, in the tail of anaesthetized rats. The advantage of this method lies in the potential for continuous evaluation of free radicals and singlet oxygen during nociceptive processes.

METHODS: Electron paramagnetic (spin) resonance (EPR/ESR) was used. DMPO and PBN as spin traps and thermal mechanical pulp (TMP) as a spin detector of singlet oxygen were used. Thirty-one adult male (Wistar) rats were used for the experiments. They were housed according to principles of good laboratory practice. The animals were stimulated for 10 minutes on 5 consecutive days by using clamps on the hind limbs. During the EPR measurement they were anaesthetized with a mixture of ketamine and xylazine. Hydroxyl and nitroxide free radicals, as well as singlet oxygen were measured.

RESULTS: After nociceptive stimulation, free hydroxyl radicals were increased as well as free nitroxide radicals. Singlet oxygen was also increased after nociceptive stimulation. Antioxidants significantly decreased the increase in hydroxyl radicals after nociceptive stimulation.

CONCLUSIONS: Our results confirmed an increase in free radicals and singlet oxygen after nociceptive stimulation and a reduced increase after application of antioxidants. Direct EPR methods were first used in the tail of anaesthetized rats and represent an extremely useful tool for the evaluation of pain intensity in living animals.

ORIGINAL ARTICLE

INTRODUCTION

The most widespread methods of measurement of pain intensity are based on subjective evaluation using the visual analogue scale (VAS) or on qualitative and semiquantitative descriptions of pain sensation included in psychological questionnaires. The neuronal unit activity is an appropriate and accurate method, but it is not possible to use it routinely in humans. Imaging methods (e.g. fMRI, PET) are extremely valuable, but still very expensive. Therefore we have directed our attention toward determining several blood biochemical parameters, which might reflect pain intensity and could be used to monitor treatment. In animal experiments, we have found that after mechanical nociceptive stimulation the final products of free oxygen radicals, thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA), are increased as well as the participating enzymes superoxide dismutase (SOD) and glutathione peroxidase (GDX). These changes were measured in the sensorimotor brain cortex [31, 35]. More detailed changes in free radicals production were detected using electron paramagnetic resonance (EPR). Reactive oxygen substances were produced in both the brain cortex and in the blood of the test animals. Changes in singlet oxygen, nitroxide, hydroxyl, and peroxyl radicals were studied and their complicated interplay was described. We found that in rats, nociceptive stimulation increases the products of lipid and saccharide metabolism, as well as lipoproteins in brain cortex and in blood plasma [36]. We studied patients with three diagnoses: acute pancreatitis, bone fractures, and cholecystitis. In patients with acute pancreatitis and fractures, glycemia, total cholesterol, HDL- and LDL- cholesterol and triacylglyceroles (triglycerides) were significantly increased, whereas in cholecystitis only C-reactive protein (as a marker of inflammation) was increased, compared to patients without acute pain [21]. These findings could be immediately applied in clinical practice.

Then we measured free radicals using TBARS, MDA, SOD, GDX and total anti-oxidative capacity.

We tested the effect of antioxidants (vitamin E, vitamin C, β-carotene, selenium and melatonin and analgesics (aspirin and morphine). Measurement of tail-flick latency was used as a clinical control [36].

There is evidence [39] of free radical generation during certain physiological reactions [12]. This phenomenon has been studied in detail in stress [25] and respiratory exacerbations. We speculated that free radicals were also generated during pain stimulation [36].

In this study of EPR spectroscopy of rats in vivo was performed before and after pain stimulation and under various conditions to determine if free radicals were generated and, if so, to determine their concentration as a function of conditions and dynamics of pain stimulation. Evidence [3] of hydroxyl radical generation during physiological processes and diseases [37] has been previously noted.

METHODS

For direct measurement free radicals it is necessary to use spectroscopy of electron paramagnetic (spin) resonance (EPR/ESR).

Elimination of free radicals

The reaction between two radicals leads to the sharing of unpaired electrons.
Free radicals are neutralized by redox reactions with antioxidants.
Free radicals can be captured by special compounds.
Free radicals are eliminated from the body in the urine.

Spin Trapping

Short lived radicals in living organisms are to be expected [8]. As a sequel spin trapping was carried out for the determination of hydroxyl, nitroxide and other radicals. We used DMPO and PBN as spin traps, and TMP as a spin-detector for singlet oxygen.

Chemicals

Spin traps DMPO (5,5-Dimethyl Pyrroline N-oxide), PBN (Phenyl-Tert-Butyl Nitrone), TMP (2,2,4,4-Tetramethyl-Piperidine) from Sigma-Aldrich; redistilled water and other common laboratory chemicals were also used (e.g. TMP (2,2,6,6 – tetrametyl-piperidin).

Electron Paramagnetic (Spin) Resonance Spectroscopy (EPR/ESR) [47]

Determination of free radicals by EPR is based on the ability of free radicals to absorb microwave energy in strong magnetic fields. EPR spectra were recorded on an Elexsys E-540 Bruker-Biospin (Rheinstetten, Germany) EPR spectrometer, magnetic fields were measured with a 1H-NMR magnetometer and microwave frequencies with a frequency counter. Measurements were carried out under the following conditions: microwave power 20 mW, modulation amplitude 0.05 mT, attenuation (gain) 10 dB, time constant 0.2 sec, scan speed 0.3 mT/min, calibration standard Mn^{2+}/ZnS and Cr^{3+}/MgO, measured at room temperature. The EPR spectra were recorded as the first derivation (in some cases as the second derivation) and the main parameters, such as g-factor values, hyperfine coupling constant A, line width Δ Hpp (peak-to-peak distance), and Δ App (peak-to-peak amplitude), were calculated according to Weil et al [47]. The WINEPR (Bruker, Rheistetten, Germany) software interface was used spectra recording, handling, and evaluation. Internet databases from NIH (Bethesda, Maryland, USA) and the University of Bristol, UK were also used [44].

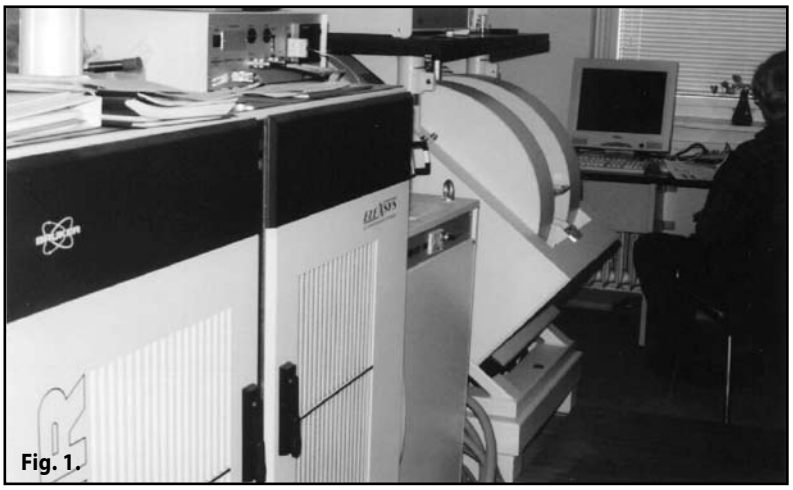

Fig. 1.

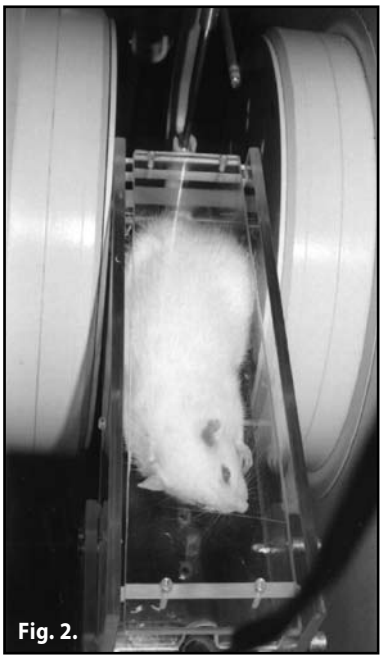

Fig. 2.

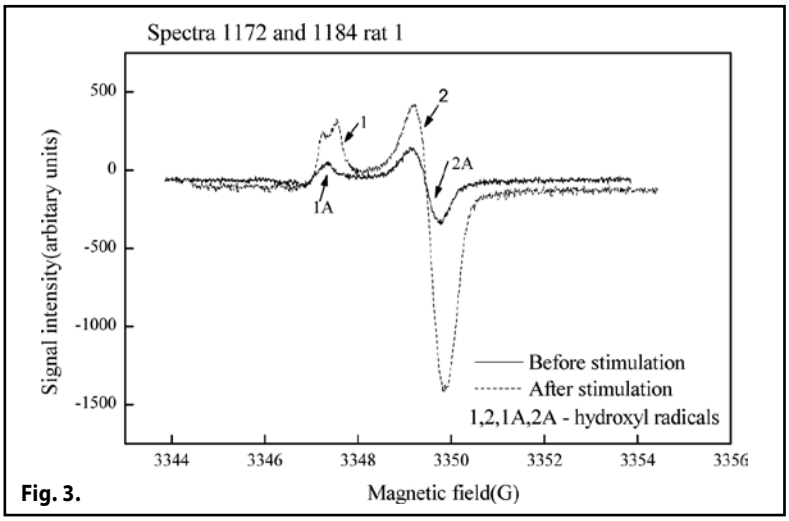

Fig. 3.

Fig. 1. EPR spectrometer of the type elexsys E-540 Bruker- Biospin.

Fig. 2. Placement of anaesthetized rat in the EPR spectrometer.

Fig. 3. Spectra of hydroxyl radicals before and after nociceptive stimulation.

Singlet oxygen $^1O_2$

In this case it was applied to rates by injection 2,2,6,6-Tetrametyl-piperidin (TMP) which is in EPR spectroscopy used selectively for identification and determination of singlet oxygen.

Singlet oxygen $^1O_2$ reacts with TMP selectively to form relatively stable nitroxide radicals; the triplet spectrum of which corresponds to the quantity of singlet oxygen present.

We acquired a similar spectrum with PBN. With regard to the selectivity of reaction, it is assumed that the increase (changes) in the measured spectrum corresponds to changes in the concentration of the generated singlet oxygen.

Method of measurement and evaluation of specimens:

The spin trap DMPO or PBN or TMP (Sigma-Aldrich) was injected intramuscularly at a dose of 0.1–0.2 ml of pure chemicals.

The gained spectra were evaluated with Bruker-Biospin EPR interface program, and figured for other uses by means of graphic program Origin. The program Bruker-Biospin enabled the direct detection of spectral constants (g-factor, amplitude of signals) ΔH_{pp}, in units of magnetic field (Gauss, event. militesla), place of signals in magnetic field and intensity of signals (A_{pp}, in work units EPR).

The signals were confronted with spectra of standards (Mn^{2+}/ZnS, Cr^{3+}/MgO, Magnettech, Berlin, Germany).

The quantitative data were obtained using double integration, by means of PC, or by spectral parameters (ΔH_{pp}, A_{pp}).

The following specimen was used:

$$N \times N_{stand.} \times (\Delta H_{pp}{}^2 \times A_{pp})_{\text{specimen}} : (\Delta H_{pp}{}^2 \times A_{pp})_{\text{standard}}$$

$N \times$ = number spins calculated

$N_{stand.}$ = number spins in standard

$(\Delta H_{pp}{}^2 \times A_{pp})_{\text{specimen}}$ = term including spectral parameters of specimen

$(\Delta H_{pp}{}^2 \times A_{pp})_{\text{standard}}$ = term including spectral parameters of standard

The evaluation method using double integration of EPR spectra was used also.

Table 1. Hydroxyl radicals

Before stimulation	After stimulation	Difference	Difference in %
376	1460	1084	388.30
235	587	352	249.79
243	438	195	180.25
344	719	375	209.01
634	2370	1736	373.82
170	415	245	244.12
1667	3785	2118	227.05
1011	1134	123	112.17
928	1525	597	164.33
2084	3709	1625	177.98
101	798	697	790.10
653	842	189	128.94
675	685	10	101.48
701.6	**1420.5**	**718.9**	**257.5**

t – value 1.98; $p = 0.6$

Table 2. Inglet oxygen

Before stimulation	After stimulation	Difference	Difference in %
173	1082	909	625.43
489	1089	600	222.70
497	1089	592	219.70
170	415	245	244.12
236	1094	858	463.56
599	737	138	123.04
435	937	502	215.40
432	737	305	170.60
498	1159	661	232.73
216	1330	1114	615.74
374.5	**966.9**	**592.4**	**313.3**

t – value 6.04; $p < 0.01$

Table 3. Antioxidants

After stimulation	After antioxidants	Difference	Difference in %
1136	632	504	–44
772	719	53	–7
283	256	27	–10
553	418	135	–24
762	407	335	–47
1124	469	665	–58
663	623	40	–6
1049	652	391	–38
792.8	**522**	**268.8**	**–29.25**

t – value 2.26; $p = 0.04$

Animals

Thirty-one adult male (Wistar) rats with an average weight of 180–220 g were used. They were bred according to the principles of good laboratory practice (alternation of 12 hours light and 12 hours darkness, food ad libitum, constant light, 8 animals per cage). All animal procedures were in strict accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the International Association for the Study of Pain [49]. All experiments were approved by the Animal Care Committee of the 3rd Faculty of Medicine, Charles University, Prague. All efforts were made to minimize animal suffering.

Painful stimulation: our own model of mechanical pain was used [34]. Clamping of both hind limbs was applied for 10 minutes on 5 consecutive days. "Crocodile" clamps were placed on the distal parts of hind limbs.

Each rat, under total anesthesia (5% ketamin at a dose of 90 mg/kg and 2% xylazine at a dose of 15 mg/kg), was placed on the plastic table of the EPR resonator and part of its tail was placed into the resonator containing a silica glass tube (Figures. 1, 2, 3).

An antioxidant mixture was applied in following concentrations: vitamin A-β karoten 0.5 mg/kg i.m., vitamin C 3.5 mg/kg i.m., vitamin E (Trolox α – tocopherol, water soluble vit. E) 5 mg/kg i.m., selenium in the form $Na_2SeO_3.H_2O$, 0.25 μg/kg i.m.

RESULTS

Statistical evaluation – Student t-test independent samples

Tables 1–3, Figures 4–6. Hydroxyl radicals: the concentration of free hydroxyl radicals increased the most.

Nitroxide radicals: after nociceptive stimulation increased by up to 10%.

Concentrations of singlet oxygen were also increased after nociceptive stimulation.

The increased hydroxyl radicals after nociceptive stimulation were significantly decreased after the administration of antioxidants.

DISCUSSION

Because free radicals can interact and change, it is necessary to accept the fact that some results differ from the average and the results have relatively large variations. Factors that can influence changes involve: possible interactions between ROS and free radicals and singlet are superoxide → peroxide → free hydroxyl radical, superoxide + nitroxide, radical → peroxinitrite mutual shifts of superoxides, free hydroxyl radical and singlet oxygen and others.

After nociceptive stimulation, hydroxyl radicals (OH) were increased as were nitroxide radicals (NO) and singlet oxygen $^1O_2$ [24]. The administration of antioxidants decreased the elevated levels of hydroxyl and

nitroxide radicals by 2/3 of the level after the nociceptive stimulation. The most dangerous free radical is hydroxyl (OH). Its half live is 10^{-9} s and acts in close vicinity to its origin. Singlet oxygen $^1O_2$ is not technically a free radical, however it behaves like them; it's a ROS (reactive oxygen species). Its half life is 10^{-6} s. The reactions of nitric oxide (NO) are somewhat different from other free radicals. It is a potent vasodilator and acts 3.5 times faster in the presence of superoxide dismutase and it has a. longer half life.

The oxidative stress

Oxidative stress arises from an imbalance between the formation of free radicals and anti-oxidative species. Free radicals can damage a variety of cell functions through lipoperoxidation, oxidation of proteins, destruction of DNA, and nitration of albumins.

As has been demonstrated several times, pain increases the formation of ROS – reactive oxygen species (singlet oxygen and hydroxyl peroxide) and free radicals (hydroxyl and nitroxide) [26]. Consequently they can lead to lesions in many tissues and can contribute to diseases [37, 12, 15, 19] such as an atherosclerosis, sarcomas, and many different neurological and psychiatric diseases [2,1, 20, 23, 27, 30, 45]. The Fenton reaction is the main source of free oxygen radicals. The free hydroxyl radical is formed from hydroxyl peroxide by the catalyzation in the presence of divalent iron and in the presence of Cu, Zn, and Al. These reactions proceed very quickly and are complete in a matter of seconds, while free oxygen radicals, formed by the Haber-Weiss reaction, are created slowly [41, 42].

There are two possible explanations which can apply in our model of pain stimulation. Free radicals can be also generated in various ways for example uric acid is generated from xantin oxidase. The necrosis of cells can create the superoxide. The direct measurement of free radicals and singlet oxygen in the tail of rats is an "in vivo" measurement and demonstrates integral-body oxidative stress caused by pain. Free radicals that enter the blood can also be detected in the tail. The positive effect of anti-oxidants reduces the quantity of free radicals and consequently reduces free radical concentrations in tissues. In previous experiments, we have shown increased formation of free radicals in the cerebral cortex homogens in experimental rats [31] and also increased amounts of free radicals in the blood of patients [18, 21, 43].

Most superoxide is generated through metabolism. We did not analyze the increase in superoxide with regard to pain because superoxide is less dangerous than hydroxyl radicals or singlet oxygen. Free radicals can induce numerous metabolic changes. The hydroxyl peroxide is reduced, especially by enzymes, such as catalase and glutathione oxidase. Hydroxyl peroxide is not a free radical, but it has similar properties and, moreover, it is able to penetrate cell membranes. However, superoxide reacts 3.5 times faster with nitroxide radical

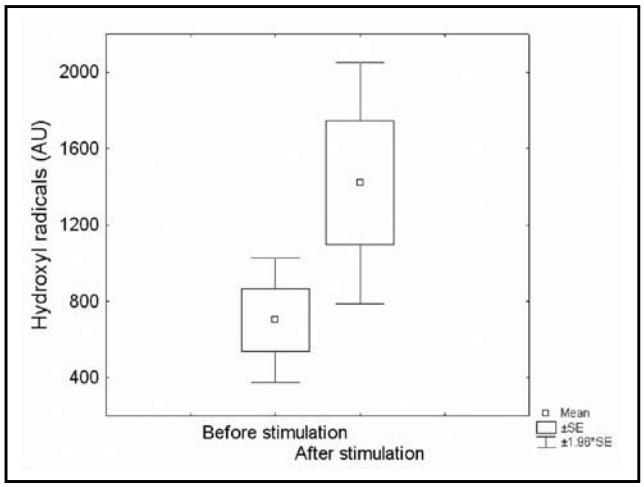

Fig. 4. Hydroxyl radicals.

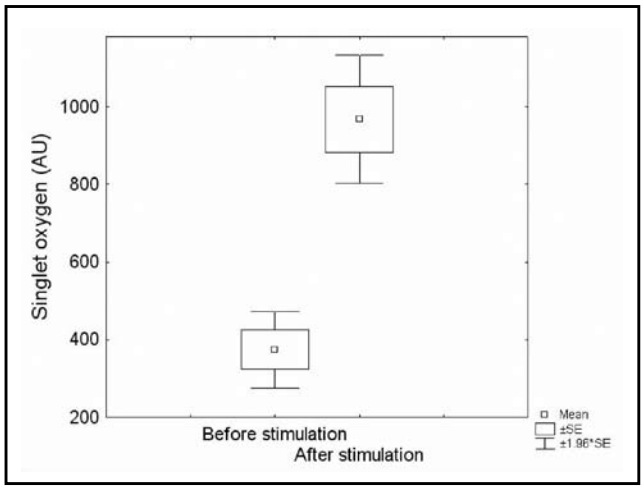

Fig. 5. Singlet oxygen.

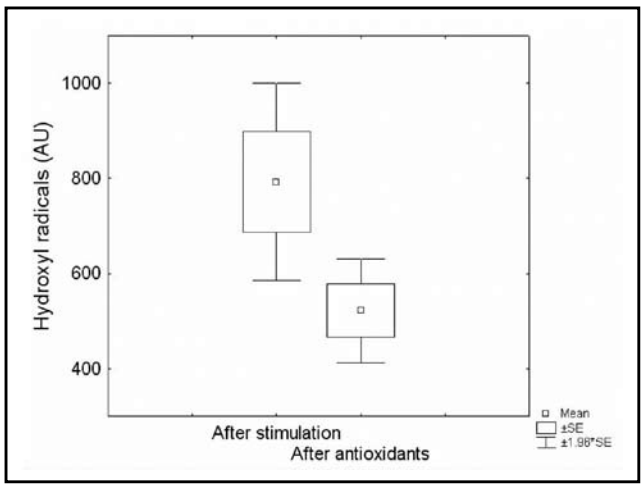

Fig. 6. Antioxidants.

and can react before dismutation into oxygen and hydrogen can be catalyzed by SOD:

$$O_2^{\cdot -} + NO\cdot \rightarrow ONOO^- \text{ (peroxinitrite)}$$

These reactions are related with nitration of different molecules.

The superoxide reacts with trivalent iron to produce Fe^{2+} reactants one molecule of oxygen. Divalent iron reacts with hydrogen peroxide to form Fe^{3+}, a hydroxide ion and a free hydroxyl radical:

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

The reaction between reduced glutathione, hydrogen ions, and superoxide form thiyol radicals and hydrogen peroxide:

$$GSH + O2^{\cdot -} + H^+ \rightarrow GS\cdot + H_2O_2$$

The 2 thiyol radicals react to form an oxidized glutathione:

$$2\ GS\cdot \rightarrow GSSG$$

hydrogen peroxide and hypochloric acid react to form a singlet oxygen:

$$H_2O_2 + HClO \rightarrow H^+ + Cl^- + H_2O + {}^1O_2$$

The singlet oxygen is highly energetic. It has 2 unpaired electrons of opposite spin in either the same or different orbits. In fact, it is not a free radical but it injures molecules in a similar manner. Singlet oxygen is created in a variety of ways, e. g. by reduction through reaction of superoxide under the influence of SOD. Superoxide reacts with free hydroxyl radicals or with 2 lipoperoxide radicals.

Free radicals are reduced also by lipid peroxidation, peroxidation of proteins, destruction of DNA, and participation in the formation of AGE (advanced glycosylation end-products).

Furthermore free radicals reduce the effect of antioxidants which markedly influence radical reactions. Free radicals can be quenched by other molecules and destroyed through the reaction of two free radicals which are dissolved together. The free radicals can also be eliminated in the urine.

It is clear from above that is difficult to monitor all the possible effects of free radicals and therefore we examined the most dangerous free radical – the (OH.), with a half-life of 10^{-9} s, and influence restricted to the neighborhood of its creation. Because pain effects the whole organisms, increases in free hydroxyl radicals can be observe in the blood of test animals. W also monitored levels of singlet oxygen which is also a very dangerous molecule. However, our anti-oxidant mixture did not contain all compounds which are not known to be effective (e. g. histidine), therefore the anti-oxidant mixture was not equally effective in all cases. Nitroxide radical increased after stimulation in only 10% of cases. [24].

Interpretation: the literature supports the idea that hydroxyl radical generation takes place in response to pain stimulation. In this case PBN was used as a spin trap. PBN was used in EPR spectroscopy to monitor hydroxyl radicals. In the control experiments we found that the EPR spectrum can be observed in laboratory animals in other ways (i.e. the measurement of hydroxyl radicals in water solution).

How to explain the changes of singlet oxygen? [38, 40]

An intense pain stimulus causes inflammation with all accompanying signs, i. e. wash out of phagocytes, activation of complement etc. [13]. Antibodies created in tissue immunocomplexes stimulate the production of free radicals and ROS (especially in phagocytes), including hydrogen peroxide, ozone and singlet oxygen [32]. A large production of ROS seems to be one of the factors which caused induction of autoimmunity associated with SLE and carcinoma. DNA isolated from the vaccine of patients with SLE and cancer inhibits anti-singlet oxygen-plasmatic DNA IgG activity [13]. It is interesting that dietetic pulps, which are offer anti-carcinoma protection for the colon, also have certain anti-oxidative capabilities [5]. In carcinoma, there is usually a lower level of uric acid which can be the result of its consumption of free radicals. Singlet oxygen is very toxic and is able to penetrate cell membranes and tissues: normally its half-life is very short (10^{-5-6} sec.). Even with 3 times its normal half-life, singlet oxygen could only travel a distance of 220 nm [33]. It is also possible that singlet oxygen reacts to form hydrogen peroxide which is also able to penetrate membranes and whose half time depends on reacting with a substrate which can oxidize it [46]. But if we consider the reaction of antibodies and the reaction with antigen, which produce singlet oxygen, then, of course, its area of action in additional places is understandable. Immediately after the pain stimulus appears there is a decrease in the level of singlet oxygen, which is a manifestation of its reactive acute consumption [17]. Inflammation develops slowly, its creation takes a certain amount of time because before the phagocytes, which produce singlet oxygen, must move into the area of inflammation, therefore singlet oxygen increase is delayed. However, during chronic inflammation, e.g. during chronic pain, stimulation singlet oxygen is already increased [6].

Reactive oxygen species (ROS) have been implicated in contributing to and/or maintaining conditions of chronic pain. To assess putative site of action, intrathecal TEMPOL (380 nmol/5 μl, i.t.) was given 5 min before intraplantar injection of formalin. Intrathecal TEMPOL produced an 83% reduction in nociceptive response in the tonic phase, but no significant attenuation of the acute phase response [10].

Malonaldehyde increased 4 days post-exercise. Ascorbic acid supplementation attenuates ROS production following downhill running. Ascorbic acid supplementation may inhibit the recovery of muscle function [4].

Reactive oxygen species (ROS) are critically involved in neuropathic pain. Repetitive daily injections of low doses of vitamin E significantly reduced neuropathic pain behaviors. Vitamin E was also effective in producing analgesia by intrathecal injection suggesting the importance of spinal mechanism [14,16].

Levels of mitochondrial reactive oxygen species increase in rat neuropathic spinal dorsal horn neurons. Reactive oxygen species (ROS) are toxic agents that may be involved in various neurodegenerative diseases. ROS are also involved in persistent pain through a spinal mechanism. The increase of mitochondrial ROS is a mechanism of central sensitization in neuropathic rats [29].

Our results indicate that patients with fibromyalgia are under oxidative stress. These findings represent a rationale for further research assessing the effect of free radical scavengers or antioxidant agents like vitamins and omega-3 fatty acids on peripheral and central mechanism in FM [28].

Stress is known to affect synaptic plasticity, dendritic morphology, and induce neurotoxic damage in humans, probably through generation of free radicals. Restraint stress induced a decrease in the level of GSH. Vitamin E was found to be most effective in restoring inherent anti-oxidant systems, no additive effect was observed in combined vitamin treatment as expected [48].

Oxidative stress is an important pathophysiological mechanism of many neurological diseases. Reactive oxygen and nitrogen species have been cited as molecules involved in the nociceptive process [9].

Increased oxidative stress resulted in an increase in the activity of anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), as observed in the cortex and striatum [7].

CONCLUSIONS

Nociceptive stimulation applied before anesthesia in rats increased the level of free radicals (OH, NO) and singlet oxygen $^1O_2$. The administration of anti-oxidants incompletely blocked this elevation.

Direct in vivo measurement of free radicals in the tail of rats confirmed our previous results which described an increase in free radical reactions (lipid peroxidation, changes of anti-oxidative enzymes etc.) after nociceptive stimulation.

In vivo EPR spectroscopy is a very suitable method for evaluating the dynamics of pain processes.

ACKNOWLEDGEMENT

This study was supported by the Research Goal 0021 6208 16, CNS 1 M 0517, CN LC 554

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