# The effect of treatment on lipid peroxidation in patients with subarachnoid haemorrhage

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## Abstract

**Introduction:** Subarachnoid haemorrhage (SAH) is accompanied by increased generation of reactive oxygen species (ROS) and intensification of the lipid peroxidation. The aim of this study was to investigate the effect of treatment on the level of lipid peroxidation products: conjugated dienes (CD) and thiobarbituric acid-reactive substances (TBARS) in SAH patients' peripheral blood plasma and erythrocytes.

**Material and methods:** The study was performed on 42 SAH patients (Hunt and Hess grade I or II) and on 20 healthy volunteers (control). All SAH patients underwent surgery and pharmacotherapy (dexamethasone, omeprazole, cefuroxime, mannitol, nimodypine). Venous blood was taken from the SAH patients before surgical and pharmacological treatment, one day after and ten days after surgery, and once from the controls.

**Results:** TBARS concentration in blood plasma and erythrocytes and CD level in erythrocytes of SAH patients before treatment was more than twice higher ($p < 0.001$) than in the controls, and CD level in blood plasma was almost three times as high ($p < 0.001$) as in the controls. One day after surgery, the concentration of these parameters decreased by about 20% ($p < 0.001$) compared to baseline level. Ten days after surgery, TBARS and CD concentration decreased two-fold ($p < 0.001$) compared to their level one day after surgery. Moreover, those values were not statistically significantly different as compared with the control group.

**Conclusions:** Surgical and pharmacological treatment of SAH patients restored prooxidant-antioxidant balance. Evaluation of TBARS and CD level, in SAH patients' peripheral blood may help to monitor the therapy efficiency in normalization of CNS oxygen metabolism.

**Key words:** conjugated dienes, thiobarbituric acid-reactive substances, intracranial aneurysm, free radicals.

## Introduction

Subarachnoid haemorrhage (SAH) is a common neurological emergency, which carries a high morbidity and mortality [1, 2]. The overall incidence of SAH is approximately 8 per 100 000 persons-years over 35 years [3], while global mortality ranges from 32-67% [4]. The main cause of SAH is the rupture of an intracranial aneurysm [1]. A consequence of SAH is often

vasospasm, which can leads to the formation of focuses of ischaemia of the brain [5]. The pathophysiology of SAH may involve reactive oxygen species production (ROS) and lipid peroxidation [6-8]. The generation of ROS and their subsequent peroxidative action on membrane polyunsaturated fatty acids (PUFA) could be enhanced after SAH [9].

One of the consequences of generating ROS is the lipid peroxidation process, which involves free radical chain reactions leading to the degradation of PUFA that build cell membranes [10]. In the lipid peroxidation process, after the hydrogen atom has been separated from the rest of the polyunsaturated fatty acid, double bonds are regrouped and conjugated dienes (CD) are formed [11]. CD are known to be the early markers of disturbance in the balance between generation of free oxygen species and the mechanisms of antioxidant defense [12]. Besides conjugated dienes, measures of lipid peroxidation include expired pentane, aldehydes, lipid peroxides or isoprostanes. Aldehydes are formed as the fatty acid is broken down during a chain of chemical reactions of lipid peroxidation. [11]. One of the more easily detectable secondary product of lipid peroxidation is malondialdehyde – MDA [7]. MDA and other thiobarbituric reactive substances (TBARS) condense with two equivalents of thiobarbituric acid to give a fluorescent derivative that can be detected spectrophotometrically. TBARS assay is the most common method used for screening and monitoring changes in MDA content [13].

Lots of the published papers concerning oxidative stress in the course of SAH involve experimental research on animals [14-16] or concern changes observed in cerebrospinal fluid [17, 18]. A small number of studies concerning changes in the concentration of lipid peroxidation products in the peripheral blood of SAH patients, although there are papers that demonstrate that oxidative stress following brain injury is not limited only to the brain. Changes in the prooxidant-antioxidant balance have also been observed in peripheral blood [19]. The aim of this study was to determine the level of CD and the concentration of thiobarbituric acid-reactive substances – TBARS (expressed as MDA concentration) – in peripheral blood plasma and erythrocytes of patients with SAH and to investigate the changes in concentration of these lipid peroxidation products after patients have undergone therapy.

## Material and methods

### Subjects

The study was performed on 42 patients with SAH (18 men, 24 women) treated in the Department and Clinic of Neurosurgery and Neurotraumatology, *Collegium Medicum* of Nicolaus Copernicus University in Bydgoszcz (Poland). The control group consisted of 20 healthy volunteers (9 men, 11 women) without any known disease, who matched by age, sex and smoking habit to the case group.

The criteria of inclusion to case group were admission of patients during the first day of haemorrhaging and surgery performed within 48 h of being admitted to the Clinic. The studied group comprised only patients whose clinical state corresponded to grade I or II on the Hunt and Hess grade. Subarachnoid haemorrhaging was diagnosed on the basis of computed tomography (CT) of the head. By using the computed tomographic angiography (CTA) option, the vascular disturbance was located. All surgery was carried out by way of the typical pterional access, finally clipping the aneurysm. The patients were also treated pharmacologically (Table I). Excluded from the study were patients who suffered from any angiographic or neurological complications and patients with inflammatory, any kind of infections or other disease that may affect prooxidant-antioxidant balance.

The studies obtained the agreement of the Bioethical Committee at the Ludwik Rydygier Medical University in Bydgoszcz. All the subjects participated in the study provided written informed consent.

### Sample preparation

Blood for analysis was taken from the antecubital vein into heparinised test tubes before surgery and before starting of pharmacological treatment (on

**Table I.** Protocol of phamacological treatment in patients with SAH

| Therapeutic agent | Before surgery | After surgery |
|---|---|---|
| Dexamethasone | 8 mg 3×/day (*i.v.*) | |
| Omeprazole | 20 mg once (*i.v.*) | |
| Cefuroxime | 1.5 g once (*i.v.*) | 1.5 g once (*i.v.*) |
| Mannitol | 100 ml 3×/day (*i.v.*) | 100 ml 3×/day (until the 5 day, gradually reducing the dose) (*i.v.*) |
| Nimodypine | | 60 mg every 4 h for the first 5 days (*o.*) |
| Compound electrolyte solution (CES) | 2 l/day (*i.v.*) | 2 l/day for the first 3 days (*i.v.*) |

the day of admission to the Clinic), one and 10 days after surgery. Blood samples were taken once from the control group. The concentration of TBARS and the level of CD in the erythrocytes and the blood plasma were determined.

In order to obtain blood plasma, the venous blood was centrifuged (5403 Refrigerated Centrifuge, Eppendorf HQ, Hamburg Germany) for 10 min at 6,000 g in temp of +4°C. After plasma removal, the erythrocytes were washed three times with solution of phosphate buffered saline (PBS). After each washing, they were centrifuged and the supernatant was removed. During supernatant removal the upper layer of leukocytes and platelet was also eliminated and the blood smears were done in order to detect the leukocyte reminiscence. The verification of protein content in supernatant was made by 20% water solution of sulphosalicylic acid. When the negative reaction was revealed, the erythrocytes were placed in PBS solution to obtain the erythrocytes suspension with haematocrit amounted to 50%.

The concentration of haemoglobin in erythrocytes was determined by the cyanomethaemoglobin method [20], using diagnostic kits from the Polish Chemical Reagent Company S.A. (Gliwice, Poland). The content of haemoglobin in the sample was expressed in g/dl.

### TBARS assay

The concentration of TBARS was determined using the method described by Buege and Aust [21] in the Esterbauer and Cheeseman modification [22].

In order to determine the concentration of TBARS, 0.5 ml haemolysate of erythrocytes or 0.5 ml blood plasma was mixed with 4.5 ml of a reactive mixture containing 0.375% TBA and 15% trichloroacetic acid (TCA) in 0.25 N HCl. To prevent the formation of lipid peroxidation products during the reaction itself, 0.01% 3.5-diisobutylo-4-hydroxytoluene (BHT) solution was added to the test tubes as an inhibitor of the lipid peroxidation process. The samples were incubated for 20 min in a water bath at a temp. of +100°C. After cooling, they were centrifuged for 15 min at a temp. of +4°C at 2000 g. The absorption of the supernatant was measured at a wavelength of $\lambda$ = 532 nm in relation to the reactive mixture incubated in the same conditions. The majority of TBARS are MDA; therefore the concentration of TBARS was expressed in nmol MDA/g Hb in erythrocytes, and in nmol MDA/ml in blood plasma.

### CD assay

The level of CD was determined after Sergent *et al.* [23]. CD form in the lipid peroxidation process, as a result of the regrouping of double bonds after the separation of the hydrogen atom from the rest of the PUFA. They give a characteristic absorbency peak at a wavelength of $\lambda$ = 233 nm.

0.5 ml chloroform was added to 0.5 ml blood plasma or 0.5 ml haemolysate of erythrocytes, centrifuged and then 0.1 ml of the solution from the bottom layer was placed into clean test tubes. The samples were evaporated in a nitrogen atmosphere, dissolved in cyclohexane and then the absorbency at a wavelength of $\lambda$ = 233 nm was noted. The concentration of CD was expressed in units of absorbency per mililitre of plasma (Abs/ml) and in units of absorbency per g Hb (Abs/g Hb).

The optical density measurements of all of the samples were done with CARY 1E UV-Vis spectophotometer controlled by the Cary WinUV software (Varian Inc., Palo Alto, USA).

The reagents that were used came from the Sigma company (Sigma-Aldrich sp. z o.o. Poland) and from the Polish Chemical Reagent Company S.A. (Gliwice, Poland).

### Statistical analysis

The date were expressed as mean ± standard deviation. The results were analysed by the one-way ANOVA test. The correlation coefficients between parameters for an evaluation of relationships were also estimated. *P* values < 0.05 was considered statistically significant.

## Results

Demographic and clinical parameters in patient and control groups were shown in Table II. The concentration of TBARS in the blood plasma and erythrocytes of patients with SAH was more than twice as high as baseline level in the control group ($p < 0.001$) (Table III). The level of CD in the erythrocytes of patients with SAH was also more than twice as high before treatment as in the control group ($p < 0.001$) and the level of CD in plasma was almost three times as high ($p < 0.001$).

One day after surgery, the concentration of TBARS and CD in both the blood plasma and erythrocytes of the patients decreased by about 20% ($p < 0.001$) compared with the concentration before treatment (Table III). The concentration of these lipid peroxidation products was higher on this day than in the control group ($p < 0.001$). On the tenth day after surgery, the concentration of TBARS and CD in both the blood plasma and erythrocytes of the patients decreased two-fold ($p < 0.001$) compared with the level of these parameters one day after surgery. Moreover, the values were not statistically significantly different as compared to the concentration of TBARS and CD in the control group. The concentration of these lipid peroxidation products were about 2-3 times lower on the tenth day after surgery than baseline levels ($p < 0.001$).

A statistically significant positive weak correlation was demonstrated between the concentration of

**Table II.** Demographic and clinical parameters in patient and control groups

| Variable | Patients with SAH ($n$ = 42) | Control group ($n$ = 20) |
|---|---|---|
| Age (± SD) | 46 ±9 | 42 ±8 |
| Male : female | 8 : 24 | 9 : 11 |
| Smoking : nonsmoking | 23 : 19 | 12 : 8 |
| Hunt and Hess grade ($n$): | | |
| • grade I | 21 | |
| • grade II | 21 | |
| Fisher scale ($n$): | | |
| • group 2 | 15 | |
| • group 3 | 17 | |
| • group 4 | 10 | |
| Aneurysm Location ($n$) | | |
| • anterior communicating complex | 20 | |
| • middle cerebral artery | 12 | |
| • internal carotid artery | 10 | |

*SD – standard deviation*

**Table III.** Concentration of thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD) in blood of patients with subarachnoid haemorrhage and in a control group

| Parameter | Control group | Case group | | |
|---|---|---|---|---|
| | | before treatment | 1 day after surgery | 10th day after surgery |
| TBARS in blood plasma ($10^{-1}$ nmol MDA/ml) | 4.0 ±0.8 | 9.5 ±1.7* | 7.5 ±1.6*[a] | 4.2 ±0.7[a, b] |
| TBARS in erythrocytes (nmol MDA/g Hb) | 37.4 ±7.5 | 91.5 ±10.9* | 74.9 ±12.2*[a] | 35.2 ±6.8[a, b] |
| CD in blood plasma ($10^{-1}$ Abs/ml) | 6.7 ±0.8 | 19.3 ±1.5* | 15.5 ±3.2*[a] | 7.9 ±1.1[a, b] |
| CD in erythrocytes ($10^{-1}$ Abs/g Hb) | 0.8 ±0.1 | 2.2 ±0.2* | 1.6 ±0.3*[a] | 0.7 ±0.2[a, b] |

*Values are expressed as mean ± SD*
**$p < 0.001$ – compared with control group, [a]$p < 0.001$ – compared with before treatment, [b]$p < 0.001$ – compared with 1 day after surgery*

TBARS in erythrocytes and CD in erythrocytes of SAH patients on the first day after surgery ($r$ = 0.311, $p < 0.05$) as well as between the concentration of CD in plasma and erythrocytes on the tenth day after surgery ($r$ = 0.365, $p < 0.05$). The correlation was also revealed between the concentration of TBARS in plasma and CD in plasma on the tenth day after surgery ($r$ = 0.661, $p < 0.001$).

## Discussion

A higher concentration of lipid peroxidation products was found in the blood of patients with SAH before treatment than in the individuals in the control group, which is the evidence of increased generation of ROS and of a disturbance of the prooxidant-antioxidant balance. The increased concentration of lipid peroxidation products in blood plasma is probably the effect of intensified peroxidation of lipoproteins of the plasma, mainly the low-density lipoprotein (LDL) fraction and lipids of neuron membranes and glia. The higher concentration of TBARS and CD in erythrocytes is the evidence of intensification of the peroxidation process in erythrocyte membranes.

ROS form during haemolysis of extravasated blood flowing into the subarachnoid space. The haemoglobin released from the erythrocytes participates in the formation of superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical –$^{\cdot}$OH [24]. The superoxide radical is formed during the spontaneous oxidation of oxyhemoglobin to methemoglobin. The reaction of dismutation of the $O_2^{-\cdot}$ generates $H_2O_2$, which can be transformed into a hydroxyl radical in Fenton's and/or Haber-Weiss's reaction. The final radical $^{\cdot}$OH is directly responsible for the initiation of the lipid peroxidation [25]. Moreover, the iron in haemoglobin is linked to lipid peroxidation process and

oxidative cell injury [7]. Both superoxide anion generated within the erythrocytes and the iron atom may be responsible for intensification of lipid peroxidation process in erythrocytes membranes. Macrophages and neutrophils also participate in the production of ROS [26].

The generation of ROS following haemolysis of a subarachnoid blood clot, including the scavenging of a vasodilator, NO, by the superoxide radical, is one of the significant mechanisms in the pathogenesis of cerebral vasospasm [14].

Brain tissue is susceptible to oxidative injury. Membrane lipids are rich in PUFA [27] such as arachidonic acid, which are especially sensitive to free radical-induced peroxidation. Lipid hydroperoxide, being a primary lipid peroxidation product, can, because of its relative stability, leak from the cells into the bloodstream and migrate to other cells, tissues and organs. This means the transference of lipid peroxidation from one site to others in our body [13]. Secondary lipid peroxidation products, including aldehydes, e.g. MDA do not generate free radicals but can also diffuse over considerable distances from the site where they are formed. They can therefore play the role of "secondary transmitters" of ROS damage [28].

Other authors also indicate the increase in the concentration of lipid peroxidation products in blood plasma after SAH. Polidori *et al.* [8] demonstrated an increase in plasma levels of cholesteryl ester hydroperoxides (CEOOH) in patients with SAH. Increased levels of CEOOH were associated with increased mortality and correlated with clinical outcome scales. The authors believe that measurements of CEOOH in plasma may be useful both prognostically as well as in monitoring therapeutic interventions. Higher concentration of MDA in patients with SAH than in controls was found in blood plasma [29], as well as in blood serum [7]. Iavorskaia *et al.* [30] observed an intensified lipid peroxidation process in blood and erythrocytes of patients with ischemic and haemorrhagic stroke, while Bolokadze *et al.* [31] revealed higher MDA content in the blood flowing out of the damaged hemisphere of the neurocritical patients (brain infarcts, parenchymatous and subarachnoid hemorrhage) as compared to the control group.

Surgical and pharmacological treatment led to a decrease in the concentration of lipid peroxidation products. A significant decrease in the concentration of CD and TBARS in both blood plasma and erythrocytes was observed after the first day after surgery had passed. On the tenth day after surgery, a further decrease in the concentration of the investigated lipid peroxidation products was observed. The concentration of CD and TBARS measured on the tenth day after surgery was similar and not statistically significantly different as compared to the level in healthy persons.

The fact that a decrease in the concentration of the investigated peroxidation products leads to a reduction in the rate of the lipid peroxidation process is testified to by the positive correlation demonstrated in this paper between TBARS in erythrocytes and CD in erythrocytes of SAH patients one day after surgery ($r = 0.311$, $p < 0.05$) and between the concentration of TBARS in plasma and CD in plasma on the tenth day after surgery ($r = 0.661$, $p < 0.001$). The observed level of MDA (representing the majority of TBARS) in blood depends on the rate of formation of MDA and how quickly it is scavenged in the liver or in skeletal muscles [32]. Changes in the concentration of MDA that are positively correlated with changes in the concentration of CD (primary peroxidation products), whose level decreases after surgery, are therefore evidence of a reduction in the rate of MDA production and not of changes in the rate at which it is scavenged.

The reduction in the concentration of lipid peroxidation products after surgery is the effect of the elimination of haemoglobin and the products of its degradation as potential sources of ROS. However, Kaynar *et al.* [7] revealed no decrease of MDA concentration in the serum of patients both after 5$^{th}$ and 7$^{th}$ day after SAH. MDA concentration remained still higher than in control and it shows a trend to increase. The observed differences in comparison to the results of our own research may be due to the application of not only surgical but also pharmacological treatment. In order to reduce the risk of vasospasm and thus prevent cerebral ischaemia and consequent events, patients were given calcium channel blocker nimodypine. Precautions against hypoxia, and consequently against reperfusion occurring subsequently, protected against the generation of ROS in the reaction catalysed by xanthine oxidase. Patients were also given dexamethasone, which reduces the symptoms of meningeal syndrome and headache. There is research in which it is demonstrated that dexamethasone diminished the level of brain regional lipid peroxidation [33]. Patients also received mannitol, a hydroxyl radical scavenger [34]. There are also some papers which point to antioxidant capacity of omeprazole [35] and cefuroxime [36]. Omeprazole was revealed to block stress-induced increased generation of hydroxyl radical and associated lipid peroxidation and protein oxidation, indicating that its antioxidant role plays a major part in preventing oxidative damage [35]. Antioxidant effect of cefuroxime action was observed *in vitro* using stimulated human polymorphonuclear neutrophils. The possible therapeutic role of this drug was postulated for

protecting of host tissues from hypochlorus acid induced oxidative damage [36].

In conclusion, the results obtained prove that SAH leads to oxidative stress, the effect of which is an increase in the level of lipid peroxidation products in the blood plasma and erythrocytes of patients. Treating SAH patients surgically and pharmacologically restored the prooxidant-antioxidant balance. Determining the level of lipid peroxidation products – TBARS and CD – in the peripheral blood of SAH patients, and also in the cerebrospinal fluid, can help to monitor the effectiveness of the applied therapy in normalization of oxygen metabolism of CNS.

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