REDOX STATUS IN PATIENTS WITH FEMORAL NECK FRACTURES

Goran Pesić1, Jovana Jeremić2, Isidora Stojić2, Aleksandra Vranić2, Marija Canković2, Tamara Nikolić2, Nevena Jeremić2, Aleksandar Matić3, Ivan Srejović4, Vladimir Zivković4, Vladimir Jakovljević4

1Orthopedic and Traumatology Clinic, Podgorica, Montenegro
2Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
3Department of Orthopedics, Clinical Center of Kragujevac, Kragujevac
4Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

ABSTRACT

The femur transfers the body weight from the pelvic bone to the shinbone. Femur fractures are a significant cause of morbidity and mortality among the group of locomotor apparatus injuries, especially in the elderly population. Considering that oxidative stress occurs as a result of increased production of free radicals that damage cell function and cause numerous pathological conditions and diseases, the aim of this study was to investigate oxidative stress parameters in older patients with femoral neck fractures. This clinical study included 70 patients, of which 35 had femoral neck fractures (26 males and 9 females), while the other half of the patients formed the matched control group. Markers of oxidative stress (NO2−, TBARS, H2O2 and O2−) and antioxidative enzymes (SOD, CAT, and GSH) were measured. Results showed that the levels of O2− increased, while levels of NO2−, H2O2 and all the antioxidative enzymes decreased in patients with femoral neck fractures. These findings indicate that fractures cause oxidative stress, probably because of the reduced activity of osteoblasts and the increased activity of osteoclasts.

Keywords: Oxidative stress, Antioxidant enzymes, Fracture, Femoral neck

SAŽETAK


Ključne reči: Oksidacioni stres, Antioksidativna zaštita, Prelom, Vrat butne kosti

ABBREVIATIONS

NO - Nitric oxide
CAT - Catalase
ROS - Reactive oxygen species
MDA – Malondialdehyde
SOD – Superoxide dismutase
INTRODUCTION

The femur is the longest, strongest and largest bone in the human body. It transfers the body weight from the pelvic bone to the shinbone (1). Femur fractures are a significant cause of morbidity and mortality among the locomotor apparatus injuries, especially in the elderly population (2). Although any part of this bone can break, the femoral neck or hip is the most common part to fracture (3). The number of femoral fractures is increasing, and this trend will continue. In particular, higher incidences of hip fractures are recorded in the Scandinavian countries, the United States and Western Europe, while the rate is much lower in the Far East and Africa (4, 5). The factors contributing to this epidemic include, the length of time since the implantation of a prosthesis, loose stems and an aging population. Because of these factors, a large number of hip arthroplasties are performed each year (6). In young patients, femur fractures occur very rarely, and when they do, they occur due to major trauma (traffic accidents, fall from heights). Femur fractures occur more frequently in the elderly (over sixty-five years), where trauma plays a less significant role but factors such as low mineral density due to osteoporosis, osteopenia, or muscle atrophy play important roles (7). The main signs of fracture are pain and functional impotence. The patient cannot actively raise either the injured limb or their heel from the ground (8).

Considering that oxidative stress occurs as a result of the increased production of free radicals that damage cell function and cause many pathological conditions and diseases, it is not surprising that in recent years, its role has been increasingly examined in a variety of fractures. Data from existing literature has shown that reactive oxygen species (ROS) in physiological conditions contribute to the destruction of calcified tissue (9). Some studies have found that increased production of free radicals is associated with reduced bone density (10). Additionally, the increased activity of osteoclasts and decreased activity of osteoblasts may contribute to the imbalance between pro-oxidants and antioxidants in patients with various broken bones (11). In a study, Yeler and coworkers have shown that the production of free radicals is highest immediately after the fracture and continues for several months during the period of healing (12).

Despite extensive research, the impact of free radicals and antioxidant enzymes on fractures and whether the ROS production exceeds the antioxidant enzyme activity has not been fully understood. Based on the above data, the aim of this study was to investigate oxidative stress parameters in older patients with femoral neck fractures.

MATERIALS AND METHODS

Subjects

The prevalence study included 70 patients, of whom 35 patients had femoral neck fractures (26 males and 9 females), while the other half of the patients were matched with regard to sex, age and other characteristics and designated as the control group. The average age of the patients with femoral neck fractures is 66 years (women - 65.9 years; males - 66.1 years). All patients with fractures were admitted to the orthopaedic ward of the Clinical Center in Kragujevac during the period from February 2015 to May 2015.

All subjects were over 60 years old, and all patients from experimental group showed indications that required surgical intervention. The exclusion criteria included a lack of thigh/lower leg, a stump unsuitable for prosthesis (a stump with trophic changes due to islands, ulcer, fistula, a painful neuroma, deformities stump, extensive scarring, or extreme muscle atrophy), damage to the spinal cord or peripheral nerve injury (quadriplegia, paraplegia and hemiplegia) with or without loss of control of urination and defecation, and tertiary stages of malignant diseases.

All patients were familiarized with the study's protocol and their written consent was obtained. The study was approved by the Ethical committee of the Clinical Center of Kragujevac, Kragujevac.

The study protocol

This was a prospective clinical study conducted from February 2015 to June 2015.

The venous blood samples from subjects in the control group were obtained only once. Blood samples were taken from the patients in the experimental group during the first 12 hours after a femoral neck fracture. All blood samples were collected from the antecubital veins into Vacutainer test tubes containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/L NaCl, and haemolysates containing approximately 50 g Hb/L were used in the determination of antioxidant enzymes.

Biochemical assays

The following markers were measured from plasma: superoxide anion radical ($O_2^{-}$), hydrogen peroxide ($H_2O_2$), index of lipid peroxidation (measured as TBARS), and nitric oxide (NO) in the form of nitrite ($NO_2^{-}$). The activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH)) were determined in the haemolysates.

Biochemical analyses of oxidative stress parameters and antioxidant enzymes were conducted in the Laboratory of Cardiovascular Physiology at the Faculty of Medical Sciences in Kragujevac. The spectrophotometric measurements were conducted using the Shimadzu UV-1800 instrument, North America.

Measurement of superoxide anion radical ($O_2^{-}$) concentration

Determination of the superoxide anion radical ($O_2^{-}$) concentration is based on the reaction of $O_2^{-}$ with nitro blue tetrazolium (NBT), which results in nitro blue forma-
The maximum absorption wavelength used for these measurements was \( \lambda_{\text{max}} = 550 \text{ nm} \).

**Determination of the hydrogen peroxide (H\(_2\)O\(_2\)) concentration**

Determination of the hydrogen peroxide (H\(_2\)O\(_2\)) concentration is based on oxidation of phenol red using hydrogen peroxide, a reaction that is catalysed by the enzyme horseradish peroxidase (HRPO) (14). This reaction results in the formation of a compound that has a maximum absorption at wavelength \( \lambda_{\text{max}} = 610 \text{ nm} \).

**Determination of the index of lipid peroxidation (TBARS)**

The levels of lipid peroxidation were determined indirectly by measuring the products of lipid peroxidation reactions with thiobarbituric acid (Thiobarbituric Acid Reactive Substances - TBARS). This method is based on the determination of the levels of one of the lipid peroxides malondialdehyde (MDA) based on its reaction with thiobarbituric acid (TBA) (15). Distilled water was used as the blank probe. Measurements was obtained at a wavelength of \( \lambda = 530 \text{ nm} \).

**Determination of nitrite (NO\(_2^–\)) levels**

Nitric oxide (\( \cdot \text{NO} \)) decomposes rapidly to form stable nitrite/nitrate metabolic products. The method for the detection of plasma nitrite levels is based on the Griess reaction. Nitrite (NO\(_2^–\)) levels were determined as an index of NO production, which reacts with the Griess reagent to form a purple diazo-complex (16). Nitrites were measured at a wavelength of 550 nm.

**Determination of superoxide dismutase (SOD) activity**

The determination of superoxide dismutase (SOD) activity is based on the epinephrine method. A mixture of 100 \( \mu \text{L} \) lysate and 1 mL carbonate buffer was prepared, and 100 \( \mu \text{L} \) of epinephrine was then added to the mixture. The detection was performed at a wavelength of 470 nm. This method belongs to the ‘negative type’ group of methods, because it monitors the decrease in auto-oxidation speed in an alkaline medium, which is dependent on O\(_2^–\) levels (17).

**Determination of catalase (CAT) activity**

The determination of catalase (CAT) activity in the sonificate is based on the methods described by Beutler (18, 19). The lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin. Then, 50 \( \mu \text{L} \) catalase buffer, 100 \( \mu \text{L} \) sample, and 1 mL 10 mM H\(_2\)O\(_2\) were added to the samples. The results were detected at a wavelength of 360 nm. Distilled water was used as the blank probe (20).

**Determination of reduced glutathione (GSH) levels**

The reduced glutathione (GSH) concentration is determined spectrophotometrically using the Beutler method (21). The absorbance (A) is measured at a maximum absorption wavelength of \( \lambda_{\text{max}} = 420 \text{ nm} \).

**Statistical Analysis**

Statistical analysis was conducted using the statistical package SPSS 20.0 for Windows. The results are expressed as means ± standard deviation of the mean (SD). The data distribution was analysed using the Shapiro-Wilk and Kolmogorov-Smirnov tests, and depending on the results, the appropriate parametric or nonparametric test was used. The alpha level for significance was set to \( p < 0.05 \).

**RESULTS**

**O\(_2^–\) levels**

The values of O\(_2^–\) levels were significantly higher in the experimental group compared to the control group (\( p < 0.05 \)) (Figure 1).

**H\(_2\)O\(_2\) values**

The values of H\(_2\)O\(_2\) levels were significantly lower in patients with femoral neck fractures compared with healthy subjects (\( p < 0.01 \)) (Figure 2).

**TBARS values**

The values of TBARS were not statistically different in the experimental group compared with the control group (\( p > 0.05 \)) (Figure 3).

**NO\(_2^–\) levels**

The values of NO\(_2^–\) levels were significantly lower in patients with femoral neck fractures compared to healthy patients (\( p < 0.01 \)) (Figure 4).

![Figure 1.](image-url)
Figure 2. The values of hydrogen peroxide levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Figure 3. The values of lipid peroxidation (measured as TBARS) in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Figure 4. The values of nitric oxide levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Figure 5. The values of superoxide dismutase levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Figure 6. The values of catalase levels in control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Figure 7. The values of reduced glutathione levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.
Values of antioxidative enzymes CAT, SOD and GSH

The values of all measured antioxidative enzymes were statistically lower in the experimental group compared to the control group (p < 0.01) (Figure 5, 6, 7).

DISCUSSION

Fracture of the femoral neck (hip) is a typical fracture in elderly patients. Despite all the surgical achievements in recent years, hip fractures cause high rates of complications and thus increase disability, morbidity and mortality. One-third of the patients require a higher level of long-term care; furthermore, the death rate in the hospitals is approximately 10%, and mortality during the first year of fracture is approximately 27% (22). As stated above, the risk of fractures is correlated with the age of the patient because any loss of osteoblasts or increase of osteoclasts leads to osteoporosis, lower bone density, decrease in bone mass and deterioration of bone, and thus results in an increased risk for fractures (9).

Considering these factors, the aim of this study was to investigate the value of the oxidative stress parameters and antioxidative enzymes in elderly patients with femoral neck fractures. We must note that many scientists believe that the damage caused by free radicals is the main factor that leads to aging of cells and tissues (23, 24). In fact, it is believed that free radicals damage cellular macromolecules, including proteins, lipids, and DNA, leading to aging and cell death. In addition, there is evidence to support that the aging of cells results in an imbalance between pro oxidants and antioxidative enzymes, such as superoxide dismutase, was associated with increased O$_2^-$ production by the osteoclasts (30, 31).

In the present study we found a significant decrease in the activity of SOD, which may be one of the potential reasons for the diminished decomposition of O$_2^-$ and, therefore, its increased release.

Unlike the O$_2^-$ levels, the levels of hydrogen peroxide are lower in patients with femoral neck fractures compared to those who do not have a fracture (Figure 2). These results are puzzling, because the activity of both the enzymes involved in the degradation of H$_2$O$_2$ (SOD and CAT) was also reduced. In the literature, there are no other studies that examine H$_2$O$_2$ levels in similar conditions, which provides us with a limited explanation of these results.

Because ROS have extremely short half-lives, they are difficult to measure directly. Instead, what can be measured are several products of the damage due to oxidative stress, as in the TBARS assay (32). TBARS assay values are usually reported in malondialdehyde (MDA) equivalents, a compound that is a result of the decomposition of polyunsaturated fatty acid lipid peroxides. The TBARS assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been criticized for its reactivity with compounds other than MDA (32). Although there has been an increase in TBARS values in patients with fractures, this increase was not statistically significant (Figure 3). Wang and colleagues also showed an increase in this parameter in elderly patients with femoral neck fractures (2). In their study, they investigated the value of TBARS in younger patients with femur fractures and have obtained results that show a statistically significant increase unlike those observed in elderly patients. The difference in average age between the patients in our study (66 years old) and their study (86 years old), is approximately 20 years, and could be one of the reasons why our results did not show a statistically significant increase in TBARS values.

The concentration of nitric oxide in physiological conditions is low (33). In our study, the measurement of NO$_2^-$ levels was lower in the experimental group (Figure 4), which may be due to the interaction of NO$_2^-$ with several free radicals (34). Sandukli and colleagues suggest that the excessive production of free radicals occurs three days after the fractures, and one of the reasons for the observed decline may be because the NO$_2^-$ levels were measured too soon (31).

However, it can also be expected that fracture causes changes in the antioxidative enzymatic system. Therefore, we investigated the activity of three major antioxidative enzymes, CAT, SOD and GSH, to create a complete picture of the redox state in patients with femoral neck fractures.

Superoxide dismutase is the most abundant antioxidant enzyme and its main role is to catalyse the neutralization of superoxide anions (35). A study that was carried out in France in the early 1990’s recorded a significant decrease in the levels of this enzyme in older patients (36), which is consistent with the theory that connects the free radi-
icals and the process of aging (23). In our study, there was a drastic decline in the SOD concentrations in the experimental group compared to the control group (Figure 5). Many researchers believe that the reason for the low concentrations of this enzyme in patients with femoral neck fractures is the excessive production of superoxide anion radicals (37, 38). Specifically, osteoclasts lead to an increased production of $O_2^-$, which is catalysed by SOD, and cause a reduction in the levels of the enzyme antioxidant defence system (34).

Moreover, CAT decomposes $H_2O_2$ to $H_2O$ and $O_2$ (29). Our results showed that there was a statistically significant decline of this enzyme in the experimental group (Figure 6). Although the literature mentions a possible decline of antioxidant enzymes during femur fractures (33), there are no studies that specifically examine the values of parameters such as the CAT and GSH levels. Glutathione peroxidase, like CAT, protects cells from hydrogen peroxide. Additionally, our results suggest that the fracture of the femur leads to a drop in GSH levels (Figure 7). However, this is not as drastic as the decrease in CAT levels. One possible explanation for the present imbalance between pro-oxidants and antioxidants in patients with hip fracture is the combined effect of the reduced activity of osteoblasts and the increased activity of osteoclasts. Decreased activity of all the measured antioxidants is difficult to explain, keeping in mind the lack of data in the existing literature. However, one of the potential mechanisms for these results could be a loss of caspase-2 activity during bone fracture, which leads to a reduction in the expression of antioxidant genes (40).

Considering all these results of the present study together may help to better understand the molecular mechanisms involved in femoral neck (hip) fractures. These findings could be of clinical interest and enable the implementation of antioxidant supplements as adjuvant therapy in these patients.

REFERENCES


