

Original Article

Linoleic acid induces red blood cells and hemoglobin damage via oxidative mechanism

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Abstract: Hidden blood loss typically occurs following total hip arthroplasty (THA) and total knee arthroplasty (TKA) and is thought to be related to free fatty acid (FFA). To study the effect of linoleic acid on red blood cells and to examine the pathogenesis of hidden blood loss in vivo, we generated an animal model by injecting linoleic acid into the tail veins of rats. We collected blood samples and determined red blood cell count (RBC) and levels of hemoglobin (Hb), as well as the oxidation and reducing agents in the blood, including glutathione peroxidase (GSH-PX), total superoxide dismutase (T-SOD), hydrogen peroxide (H₂O₂), and ferryl hemoglobin (Fe⁴⁺ = O²⁻), which is generated by the oxidation of Hb. Hidden blood loss occurred when linoleic acid was administered at a concentration of 60 mmol/L; RBC and Hb levels were significantly reduced by 24 h post-injection. This was followed by erythrocyte deformation, reduced activity of GSH-PX and T-SOD, and decreased levels of H₂O₂. This was accompanied by an increase in ferryl species, which likely contributes to oxidative stress in vivo. Our findings suggest that linoleic acid enhances acute red blood cell injury. Hb and RBC began to increase by 72 h, potentially resulting from linoleic acid metabolism. Thus, elevated levels of linoleic acid in the blood cause acute oxidative damage to red blood cells, eventually leading to partial acute anemia. These findings highlight the pathophysiology underlying hidden blood loss.

Keywords: Linoleic acid, free fatty acid, erythrocyte damage, oxidative stress, hidden blood loss

Introduction

FFA, metabolites of fatty emboli, stimulate neutrophils to produce reactive oxygen species (ROS), such as superoxide anion radical (O²⁻), hydroxyl radicals (OH), and hydrogen peroxide. These reactive oxygen species can damage the cell membrane via the oxidation of unsaturated fatty acids [1, 2]. For example, hydrogen peroxide (H₂O₂) has been shown to penetrate the blood cell membrane and directly oxidize hemoglobin (Hb) to ferryl hemoglobin (ferryl Hb), which is incapable of carrying oxygen [3]. Thus, this enhances anoxic damage to red blood cells. Hidden blood loss (HBL) typically occurs following artificial joint replacement. HBL often leads to post-operative anemia even after autologous or allogeneic blood transfusion in accordance with visible blood loss volume. Despite this, the underlying disease pathogenesis is still unclear [4]. Large amounts of fat can be forced into the medullary canal of the femur during artificial joint replacement. Excess fat in

the marrow can extend into the circulation, resulting in high levels of free fatty acids in the blood [5]. FFA may be associated with acute red blood cell injury. Here, we tried to investigate the relationship between hidden blood loss and free fatty acids. Linoleic acid is a critical component of polyunsaturated fatty acids. To study the effect of linoleic acid on red blood cells and to examine the pathogenesis of hidden blood loss in vivo, we generated an animal model by injecting linoleic acid into the tail veins of rats, noted changes in levels of hemoglobin and red blood cell count, and establish that FFA is associated with the pathogenesis of hidden blood loss.

Materials and methods

Animal model preparation

Fifty male Sprague-Dawley rats weighing 200 ± 20 g were purchased from Nanjing University and housed at a set temperature (24°C) in a

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humidity-controlled room with a 12 h light/dark photoperiod. Animals were allowed access to food and water ad libitum. Animal welfare and experimental procedures were carried out strictly in accordance with the care and use of laboratory animals (National Research Council, 1996). All the animals were well regulated and animal ethics were approved in this research. All experiments were performed in accordance with the guidelines approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Nanjing University. All animals were anesthetized with ether inhalation before operation.

Instruments and reagents

Instruments: hematology analyzer (SYSMEX XE-5000, Japan), centrifuge (Hermle Universal Centrifuge Z323, German), microplate reader (BIO-RAD680, USA), polarizing microscope (NIKON ECLIPSE 50I, Japan), spectrophotometer (Hewlett Packard 8453 UV-visible diode array spectrophotometer, USA).

The concentration of H_2O_2 and the activities of glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) were determined with commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Linoleic acid was purchased from Sigma-Aldrich (St. Louis, USA).

Experimental protocol and drug

Rats were randomly assigned to one of five groups ($n = 10$ per group), including one control group and four linoleic acid (LIN)-treatment groups. Linoleic acid was dissolved in a 20% ethanol solution. The control group (C) was injected with ethanol alone (0.5 ml, 20%) by intravenous administration into the tail vein. The experimental groups were treated with different concentrations of linoleic acid diluted in 20% ethanol and divided into the following groups: LIN-A (0.5 ml, 15 mmol/L), LIN-B (0.5 ml, 30 mmol/L), LIN-C (0.5 ml, 60 mmol/L), and LIN-D (0.5 ml, 90 mmol/L). Injections were performed as they were in the control group. Of course, we tried to slow down the rate and minimize concentration of administration to avoid unnecessary deaths, however, the experiment showed that half of the mortality rates in dosing concentration 90 mmol/L, which deter-

mined the upper limit of the administration concentration. The condition of the animals was continuously monitored until all rats completely recovered from anesthesia. We sacrificed these rats by spinal dislocation method when they suffered from several symptoms, including dyspnea, cyanosis, haemoptysis labiali, and convulsions. Apart from this, the rats had no special discomfort throughout the experiment.

Samples of blood (0.5 ml each time) were obtained from the caudal vein at the beginning of the treatment and then again 24, 48, and 72 hours after administration. RBC and Hb values were determined using a hematology analyzer. Morphological changes were observed after Wright's staining using a polarizing microscope. All remaining blood samples were used to measure H_2O_2 concentration and GSH-PX and T-SOD activities using a spectrophotometer; for each of these measurements, absorbance values were determined at 405 nm, 412 nm, and 550 nm, respectively, according to the manufacturer's instructions. All samples were processed within two hours after blood samples were collected. Spectral changes of Hb in the LIN-C group were typically measured with a spectrophotometer. Hb (10 mM) was mixed with buffer (0.1 M sodium phosphate) containing 100 mM DTPA. All procedures were performed at 25°C [6].

Statistical analysis

All calculations and statistical analyses were performed in SPSS for Windows (Version 19.0). Values are expressed as mean \pm SD and analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's t-test. In all cases, $P < 0.05$ was regarded as significant.

Results

Administration of linoleic acid at a concentration of 60 mmol/L resulted in significant changes in both RBC and Hb levels compared to controls (**Figure 1**). These findings confirm successful establishment of an in vivo hidden blood loss model. RBC and Hb increased in a linoleic acid dose-dependent manner. However, in the LIN-D group, half of the rats died within five minutes of administration. Prior to death, these rats suffered from several symptoms, including dyspnea, cyanosis, haemoptysis labiali, and convulsions; pulmonary embolism was considered to be the main cause of death [7].

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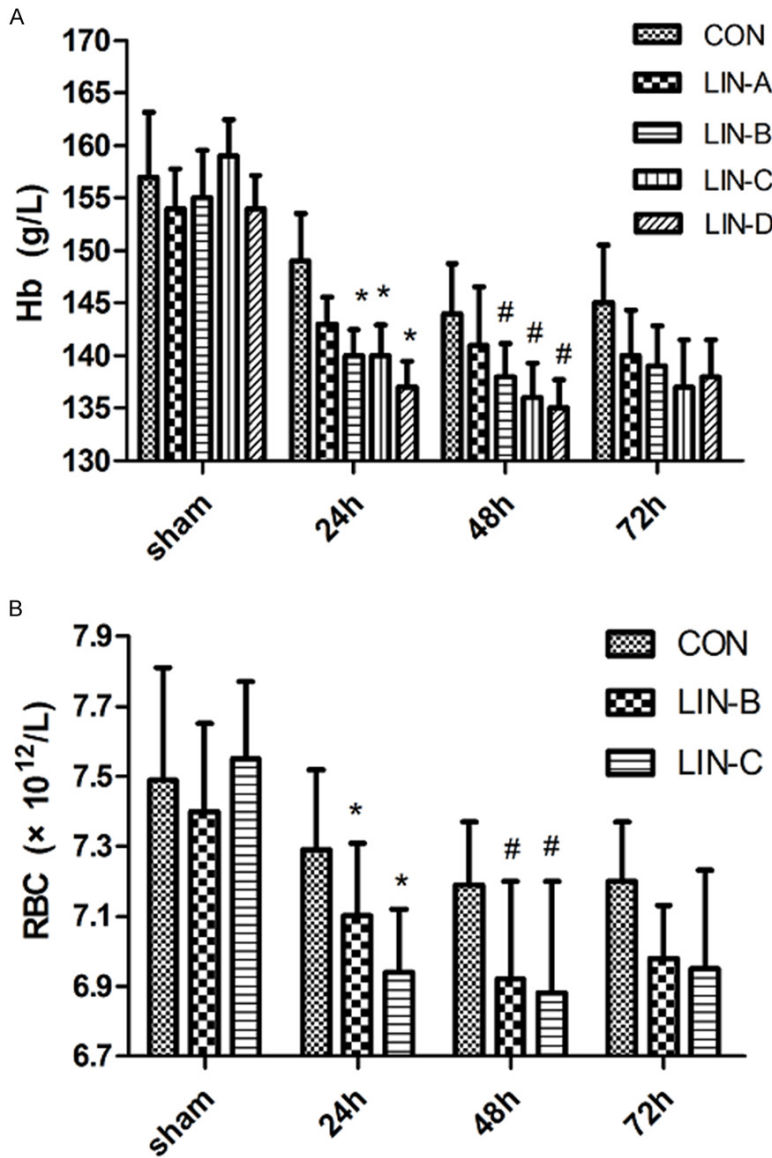


Figure 1. Changes in Hb and RBC between control group and the experimental group. Data are shown as means \pm SE; * $P < 0.05$, # $P < 0.01$.

We further analyzed groups LIN-B and LIN-C in comparison to the control. Before beginning the experiment, there were no significant differences in blood index or weight among these three groups. RBC and Hb had decreased to different extents 24 h after administration (Figure 1B). The Hb and RBC values in CON group decreased by 7.5 ± 2.3 g/L, $0.22 \pm 0.05 \times 10^{12}$ /L. In contrast, levels in the LIN-B group decreased by 13.5 ± 2.0 g/L, $0.45 \pm 0.15 \times 10^{12}$ /L, and those in the LIN-C group decreased by 16.8 ± 1.5 g/L, $0.71 \pm 0.18 \times 10^{12}$ /L. Changes between the experimental group and the

control group were statistically significant ($P < 0.05$). Activities of GSH-PX and T-SOD and the concentration of H_2O_2 decreased in all groups (Figure 2; $P < 0.05$).

Differences between groups LIN-B and LIN-C and the control were still apparent 48 hours post-injection. This was also true for GSH-PX and T-SOD activities, as well as the concentration of H_2O_2 ($P < 0.05$). At 72 h, RBC and Hb were relatively stable, and GSH-PX and T-SOD activities and the concentration of H_2O_2 were increased.

Red blood cell morphology was examined under a polarizing microscope. All blood samples were immediately mounted and stained with Wright's stain. Compared to the control group, there were several distorted red blood cells in groups LIN-B and LIN-C, and the most distinct pathological changes occurred on the first day. We identified cells with spindle and oblong shapes, pleomorphism, shrinkage, deformation, rupture, and breaking (Figure 3F, 3G).

The presence of ferryl Hb, formed by reacting with H_2O_2 , was confirmed by its reaction with sulphide ions producing a characteristic absorbance band around 620 nm [8]. The effect of linoleic acid on the hemolysis of red blood cells, either on its own or in conjunction with ROS, can be used to evaluate the degree of oxidation injury of erythrocytes [9]. We collected blood samples from the LIN-C group before administration and then every 24 hours thereafter. Absorbance peaks were detected at approximately 425 nm, corresponding to the ferryl Hb Soret peak (Figure 4).

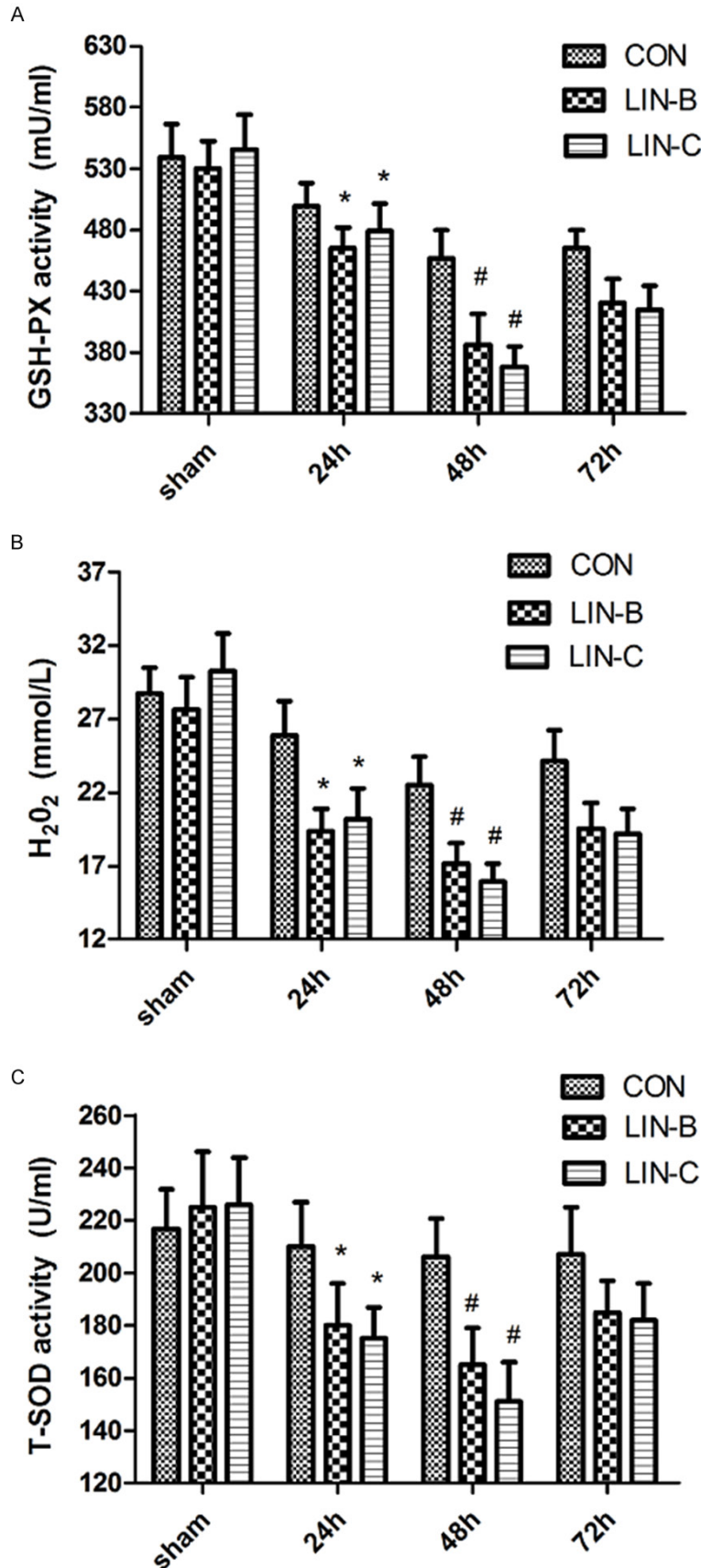


Figure 2. Changes in GSH-PX, T-SOD activity and H₂O₂ between control group and the experimental group. Data are shown as means ± SE; **P* < 0.05, #*P* < 0.01.

Discussion

Oxidative stress resulting from chronic renal insufficiency and chronic parasitic infection can lead to chronic anemia. This is often attributed to free fatty acid, which can induce chronic oxidative damage of red blood cells [10]. Clinical studies have found that levels of free fatty acid are significantly increased after total hip arthroplasty and total knee arthroplasty. Additionally, these procedures are associated with significant blood loss and post-operative anemia, which is difficult to correct even with a transfusion [6, 11]. Here, we provide evidence that free fatty acids can induce acute red blood cell damage, which is closely associated with hidden blood loss.

There are multiple theories concerning the causes and underlying mechanisms of hidden blood loss. Sehat et al [12] speculated that the recessive bleeding, at least part of the loss, is the result of hemolysis; however, the cause of the hemolysis is not clear. Haien's [13] study found that autologous blood transfusion can cause hemolysis. Specifically, when 1.3 L was auto-transfused, plasma hemoglobin levels reached 50 mg/L. At this concentration, the hemoglobin level was sufficient to cause

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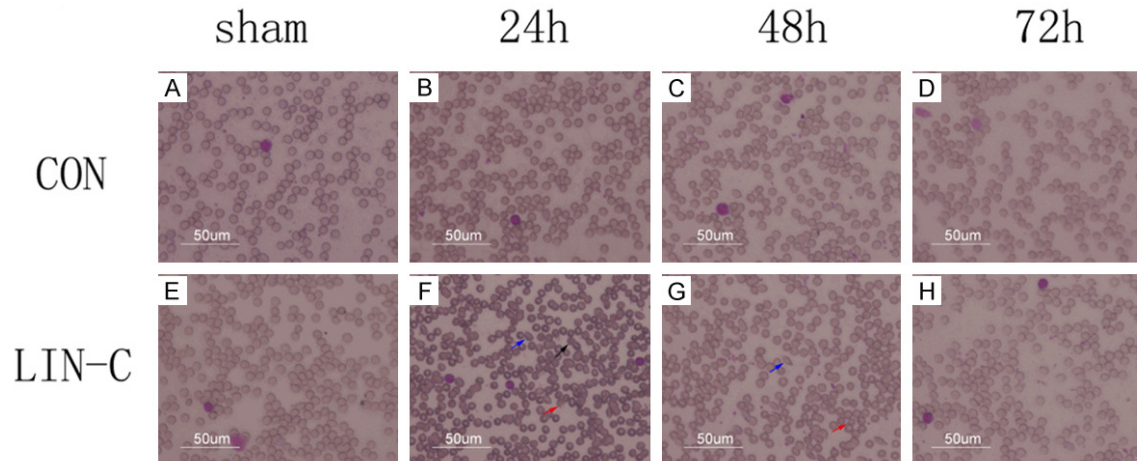


Figure 3. Effect of linoleic acid on red blood cells. Blood samples were collected before administration and then every 24 hours thereafter. Stains were added to the blood smear to observe erythrocyte morphological changes. Images are 400 \times . Cell morphology was not obviously changed in the control group (A-D); in contrast, cells in the LIN-C group (E-H) treated with linoleic acid (60 mmol/L) had a large number of red blood cells shrunken (black), deformed (blue), and even ruptured (red).

hemolysis and may also result in insufficient autologous blood transfusion. Shen et al [14] reported no significant differences between the autologous blood transfusion group and the allogeneic transfusion group in terms of hidden blood loss. However, autologous blood transfusion is a safe and effective way to decrease the use of allogeneic blood and avoid complications associated with its transfusion. Tetro et al [15] reported that it is important to use a tourniquet, which may cause the HBL. Their study showed that patients who did not receive a tourniquet during surgery were 16% lower than the user, but still reached the absolute value of the HBL (600 ml). The “third mesoecium” theory has also tried to explain this phenomenon. Using labeled red blood cell. Hou et al [16] observed a large number of labeled red blood cells in tissue clearance by using radiolabeled red blood cells after the operation, instead of using them in systemic circulation. This led to further reduced levels of hemoglobin. This may be associated with bone marrow fat, bone cement, and bone debris, which may leak into the bloodstream during operation. This can cause an abnormal capillary bed to open. However, there is no hard evidence to fully support this claim. Despite these findings, additional work is needed to fully understand the underlying mechanisms governing hidden blood loss.

Here, we have established a rat model of elevated free fatty acid by tail vein injection of lin-

oleic acid. Red blood cell damage was assessed by erythrocyte number, the content of hemoglobin, and red blood cell morphology. We also examined the activities of GSH-PX and T-SOD and levels of H_2O_2 and ferryl Hb. The data we provide here helps establish mechanisms governing hidden blood loss.

Serial determination of red blood cell count and hemoglobin levels is a good indicator of the degree of red blood cell damage. RBC and Hb levels were reduced in all treatment groups in this study. Changes in the LIN-C group were more significant than in the LIN-B group, suggesting that linoleic acid causes red blood cell damage and may play an important role in hidden blood loss.

We did notice some slight changes in the control group. This may be due to several reasons. First, blood samples (0.5 ml each time) taken from rats, with a volume extraction ratio between 2.8%-4.2%, had a direct influence on erythrocyte number, resulting in a decrease in RBC. Second, intravenous injection of the ethanol diluent also had an effect on red blood cells, the results obtained can be (at least in part) explained by the ethanol toxic effects rather than by physiological responses, including changes to membrane permeability; this could lead to erythrocyte volume increase and subsequent lysis [17]. Third, the operation itself may have induced a systemic stress response, which would change the basal metabolic rate

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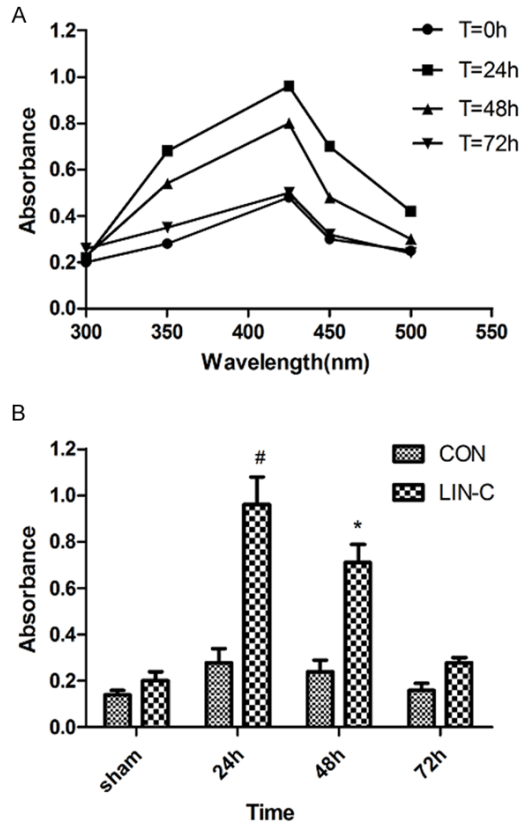


Figure 4. A. Effect of linoleic acid on the catalyzed formation of ferryl Hb. The LIN-C group absorbance changes were determined using a spectrophotometer. Measurements were taken every 24 h. For clarity, only a small subset of the spectra collected is shown, and the peak at 425 nm corresponds to a ferryl Hb Soret peak. B. Statistic analysis of absorbance changes between control group and LIN-C group, and the spectral change at 425 nm. Values are expressed as mean \pm SD (n = 5) * P < 0.01, # P < 0.001.

and cause oxidative damage to red blood cells in vivo [18]. These same considerations can be applied to the experimental groups as well. Despite these small changes in the control, it is important to note that the changes in the treatment groups were more significant. Thus, linoleic acid likely plays a critical role in this process.

Linoleic acid is a critical component of polyunsaturated fatty acids. It contains unsaturated double bonds that are highly vulnerable to free radical attack and oxidation [19]. If ROS are produced in great quantities, the body can no longer efficiently remove them. In such instances, ROS may be released into the extracellular space, causing damage to surrounding cells

and tissues [20]. Linoleic acid is a decomposition product of a free fatty acid and has been linked to erythrocyte damage.

Glutathione peroxidase is an enzyme that scavenges free radicals and catalytically breaks down hydrogen peroxide. These functions help glutathione peroxidase protect cell membrane structure and function from damage [21].

Glutathione peroxidase activity reflects the extent of lipid peroxidation; it is also an indirect measure of the severity of tissue damage caused by free radicals [22]. High levels of FFA in the blood inhibit oxidized glutathione from converting to its reduced form, thereby slowing the rate of hydrogen peroxide elimination [23]. Twenty-four hours after administration, we find that GSH-PX and T-SOD activities and H_2O_2 concentration are decreased in all groups. The changes in the experimental treatment groups were more significant, suggesting that linoleic acid plays an important role in promoting oxidation reactions in the body and decreasing GSH-PX activity. Changes in the control group may be due to stress from the blood collection procedure. Interactions between corticotrophin releasing hormone and the adrenal cortex can be enhanced by oxidative stress. An excited hypothalamic-pituitary-adrenal axis would promote secretion of adrenocorticotrophic hormone releasing hormone; this could excite pituitary ACTH neurons and increase levels of ACTH and glucocorticoids [24]. Glucocorticoids promote gluconeogenesis, which supports fat mobilization of catecholamine. They can also improve the sensitivity of blood vessels to catecholamine. Additionally, glucocorticoids help stabilize lysosomal membranes and accelerate oxidation; In contrast, GSH-PX has strong antioxidant properties. The increased concentration of linoleic acid in the blood stimulates neutrophils to produce reactive oxygen species. Moreover, catabolism of linoleic acid exacerbates the oxidation reaction and causes damage to red blood cell membranes [25]. Permeability changes in the membrane were demonstrated as morphology changes and as red cell debris. Taken together, our data show that GSH-PX activity is significantly decreased in our linoleic acid treatment groups.

Superoxide dismutase (SOD) is a ubiquitous antioxidant enzyme found in aerobic organisms. It plays a critical role in maintaining oxida-

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tion/anti-oxidation balance in the body. It helps remove superoxide free radicals from the body and protects against the toxic effect of oxygen free radicals [26]. SOD in the blood has a specific inhibitory effect on superoxide anion (O_2^-). Determining T-SOD activity can be used to assess the organism's ability to scavenge oxygen free radicals [27]. FFA can stimulate neutrophils to produce hydrogen peroxide and hypochlorous, which oxidize and consume SOD, GSH-PX, and the total antioxidant capacity of red blood cell membranes; as a result, hemoglobin is often oxidized or degenerated. Activity of both GSH-PX and T-SOD decreased by 48 h after linoleic acid injection. H_2O_2 can be broken down by Mn-superoxide dismutase (Mn-SOD) or by Zn-superoxide dismutase (Zn-SOD); it could be directly broken down by GSH as well [28]. Indicators of healthy red blood cells dramatically decreased within the first 24 h of injection, which then began to level off by 72 h. Thus, high levels of linoleic acid cause damage to red blood cells. This results in a series of redox and stress defense reactions by stimulating the blood system. Next, linoleic acid is broken down, and this catabolism has an irritation effect on the body. This eventually clears, and the hemoglobin content and red blood cell count are slowly restored as GSH-PX and T-SOD activities reach equilibrium.

Oxidative damage to hemoglobin alters its structure and function, causing it to denature and precipitate; the resulting product is called methemoglobin [29]. Hydrophilic hydrogen peroxide can directly penetrate the red blood cell membrane and oxidize hemoglobin to ferryl hemoglobin (ferryl Hb) [30]. The oxidation of heme proteins to the ferryl species by peroxides is widely considered to be the initiator of many lipid peroxidation and lipid pseudo-peroxidase reactions [31]. Hypochlorous acid oxidizes glutathione and membrane proteins-SH groups, and increases osmotic fragility. It also causes deformation of the cell membrane through lipid oxidation [3]. Studies have demonstrated that ferrous hemoglobin can be oxidized to ferryl Hb by hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL), and ferryl Hb loses the capacity to carry oxygen. However, glutathione-glutathione peroxidase (GSH/GPx) can reduce the formation of methHb by 93% when Hb is oxidized by H_2O_2 [32]. This highlights the critical role of linoleic acid in mediating Hb

oxidation and subsequent cross linking of the oxidation-reduction reaction.

In conclusion, linoleic acid induces red blood cell damage by promoting redox reactions, and more work is needed to better understand the exact underlying mechanisms. For example, there is no range of analysis and the doses used gave rise to levels (not measured) out of scale with the physiological phenomenon studied, the use of antioxidants before and after the addition of linoleic acid to the SD rats should be compared. Clinic studies should also be performed to confirm that FFA is the primary cause of hidden blood loss following THA and TKA. The data we present here also suggests that antioxidant therapy may help reduce hidden blood loss following an operation. A combination of additional clinical and pre-clinical studies will help elucidate the mechanisms responsible for hidden blood loss.

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Disclosure of conflict of interest

None.

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