

## Article

# Effect of Near-Infrared Blood Photobiomodulation on Red Blood Cell Damage from the Extracorporeal Circuit during Hemodialysis In Vitro

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**Abstract:** The contact of blood with the bioincompatible membranes of the dialyzer, which is part of the extracorporeal circuit during hemodialysis (HD), causes upregulation of various cellular and non-cellular processes, including massive generation and release of reactive oxygen species (ROS), (which is one of the primary causes of anemia in chronic renal failure). We hypothesize that near-infrared (NIR) radiation possesses antioxidant properties and is considered to protect the red blood cell (RBC) membrane by enhancing its resilience to negative pressures. Our experimental setup consisted of an HD machine equipped with a dialyzer with a polyamide membrane; whole bovine blood was examined in vitro in blood-treated circulation. Blood samples were taken at 0, 5, 15, and 30 min during the HD therapy. We also assessed osmotic fragility, hematocrit, hemolysis, and oxidative stress as a concentration of reactive thiobarbituric acid substances (TBARS). Our results have shown that RBC membrane peroxidation increased significantly after 30 min of circulation, whereas the TBARS level in NIR-treated blood remained relatively steady throughout the experiment. The osmotic fragility of NIR-irradiated samples during dialysis was decreased compared to control samples. Our studies confirm that in vitro, blood photobiomodulation using NIR light diminishes oxidative damage during HD and can be considered a simultaneous pretreatment strategy for HD.

**Keywords:** photobiomodulation; hemodialysis; blood; red blood cells; oxidative stress; low-level light therapy (LLLT); NIR radiation; reactive oxygen species (ROS)



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## 1. Introduction

Hemodialysis (HD) is the most used medical technique aimed at removing metabolic products from the blood of patients with acute and chronic renal failure (CRF) [1]. HD is generally divided into periodic treatment when the patient suffers from acute renal failure and continuous therapy for patients with CRF. Dialysis time is selected individually for the patient and is on average up to 4–5 h, three times a week [2]. The contents of soluble compounds in blood plasma are modified during HD by exposing it to dialysis fluid, with plasma and dialysis fluid separated by a semi-permeable membrane. In general transport of substances through the dialyzer membrane take place on the principle of two physical phenomena: diffusion and ultrafiltration. One of the essential components of the HD setup is a dialyzer that performs the excretory and regulatory function of the nephron. The patient's blood flows inside the capillaries, while from the outside they are washed in the opposite direction by dialysis fluid. The opposite direction of blood flow and

dialysate allows maintaining the maximum concentration gradient along the entire length of the membrane.

Anemia is a common complication that occurs in patients undergoing HD. In patients with CRF, it is mainly caused by inhibition of erythropoietin production by damaged kidneys, iron deficiency, and red blood cell (RBC) destruction [3]. Furthermore, the RBC of dialysis patients with anemia tends to live for a shorter time in the bloodstream than in healthy subjects, so the replacement of the RBC in the circulation is also impaired [4,5]. The underlying kidney disease, malnutrition, oxidative stress, dialyzer membrane biocompatibility, residual blood left in the extracorporeal circuit, and mechanical damage lead to a visible reduction in the RBC lifespan [6].

It is well established that HD constitutes a state of inflammation and excessive oxidative stress. Several reasons contribute to the formation of a high number of reactive oxygen species (ROS) in patients with HD. Some of the factors are closely related to the stage and course of the CRF and the patient's concomitant diseases (e.g., dyslipidemia, atherosclerosis, chronic inflammation, impairment of the antioxidant system, etc.). In contrast, others are strictly linked with HD procedure per se (e.g., blood biomaterials and blood dialysate fluid interactions, loss of antioxidant molecules, anticoagulation, etc.) [7]. In the last case, the interaction of blood with the dialyzer's bioincompatible membranes activates various cellular and noncellular processes, resulting in massive production and release of ROS due to the activation of white blood cells within minutes after start of HD and accumulates up to the end of the treatment. Pronounced oxidation of proteins, carbohydrates, and lipids leads to lipid peroxidation and the accumulation of advanced glycation end products that cause severe tissue damage [7,8].

Moreover, elevated oxidative stress in uremic patients is one of the main factors contributing to CRF anemia, malnutrition,  $\beta$ 2-microglobulin amyloidosis, and increased cardiovascular risk [7–11].

Comparing the contribution of mechanical damage with that resulting from biochemical changes caused by extracorporeal circulation such as HD, it can be concluded that it is mild [6]. Nevertheless, it should be emphasized that the critical role in platelet activation plays shear stresses, that is, stresses of the cell membrane that arise due to forces exerted by blood flow. Under the influence of shear forces, von Willebrand factor combines GpIIb-IIIa and GpIb/IX/V glycoproteins leading to platelet activation [12]. In addition, mechanical stresses can destroy RBC. Depending on the strength of the stimulus, the duration of its action [13] and the physiological state of the cell [14], hemolysis can occur immediately after its action, or with some delay [13]. Non-physiological flow conditions cause negative changes in the mechanical properties of RBCs, leading to a decrease in their deformability and surface charge. Even small concentrations of free hemoglobin (fHb) in plasma significantly increase RBC aggregation under conditions where small shear forces act [14,15]. A decrease in membrane elasticity and an increase in aggregation of RBCs results in an increase in blood viscosity and thus an increase in blood capillary pressure [16]. The increase in peripheral resistance of blood vessels is also the binding of nitric oxide to fHb (after depletion of the haptoglobin stock) [14]. An obvious consequence of RBC damage is decreased ability to transport oxygen to tissues.

Contemporary attempts to reduce oxidative stress and RBC damage during HD rely mainly on therapeutic interventions with antioxidants (through oral supplementation or dialyzer membrane coatings with vitamin E), optimization of the dialysis procedure or the use of ultrapure dialysate [7]. Here, for the first time, we employed photobiomodulation (PBM) to achieve this goal.

Light affects the growth and metabolism of living beings, ranging from simple single-celled microorganisms to multicellular plants and mammals, bringing beneficial effects [17]. At the beginning of the twentieth century, the dynamic development of technology allowed new light sources (e.g., fluorescent and quartz lamps). Early light applications for therapeutic purposes included skin conditions (including ulcers), syphilis, lupus, pellagra, tuberculosis, and difficult-to-heal wounds. These types of therapies involved the applica-

tion of UVA radiation in diseased areas, often in combination with the addition of plant extracts containing psoralen as photosensitizing [18]. The problem of the interaction of light with human and animal cells and tissues remained unexplored for a long time. The real breakthrough was the construction of the laser. In 1967, Endre Mester of Semmelweis University, Budapest, Hungary, experimented to see if laser radiation could cause cancer in mice. He shaved the animals' hair on the back, divided it into two groups, and exposed one of them with a ruby laser (694 nm). To the author's surprise, it turned out that light did not cause cancer and even accelerated hair regrowth. He referred to the observed phenomenon as laser biostimulation [19,20]. Along with dispelled doubts about the effectiveness of light on animal organisms, the question arose about how light interacts at the molecular and cellular levels. It was a new impulse for numerous studies and studies [20], thanks to which lasers were quickly used in physical therapy and surgery [21,22].

In clinical practice, light for therapeutic purposes is mainly used in: (a) photodynamic therapy (PDT), based on its action on a phototoxic reaction due to the action of photosensitizing substance (accumulating in cancer tissue) and light; (b) UVA radiation therapy (psoriasis); and (c) PBM, also referred as low-level light (or laser) therapy (LLLT). PBM is commonly used in physiotherapy (acute and chronic musculoskeletal pain), dentistry (inflammation and mouth ulcers), dermatology (edema, poorly healing ulcers, burns, rashes), rheumatology (chronic inflammation, autoimmune diseases) and in many other fields of medicine. It plays a particularly important role in effective pain relief and regeneration methods in sports medicine centers and rehabilitation clinics [21,23,24].

Our previous studies showed that near-infrared (NIR) radiation stabilizes RBC by increasing its resistance to destructive factors and has antioxidant properties [25–29]. Furthermore, NIR blood PBM with polychromatic light reversibly inhibits platelet activation in a dose-dependent manner. At the same time, discontinuation of the PBM leads to the cessation of the effect on platelet aggregation [29]. Our recent animal studies proved that it could be applied successfully during extracorporeal circulation using an artificial heart-lung machine to prevent platelet activation and blood destruction [30,31].

So far, it has been demonstrated in a randomized controlled trial that PBM applied in the lower limb improves functional capacity and muscle strength of CRF patients undergoing HD [32]. In another clinical trial, PBM effectively improved hyposalivation and urea levels in the saliva of these patients [33]. In this work, for the first time, we studied the effects of NIR blood PBM on oxidative stress development and RBCs destruction during *in vitro* HD. The present manuscript describes the PBM protocol, an experimental setup and an extracorporeal circulation system enabling *in vitro* HD. Experiments to test PBM on ROS formation and level of RBC destruction are presented in the results section. Finally, the discussion section summarizes our results, compares them with existing research, and provides a mechanistic interpretation, future directions, and applications.

## 2. Materials and Methods

### 2.1. Blood Preparation and Exposition to NIR Radiation

Experiments were carried out on bovine blood samples, heparinized (5 IU/mL, Polfa, Warsaw, Poland), obtained from Pro Animali (Wrocław, Poland). Blood sample was divided into two equal parts—one was used as a control test and the other was exposed to NIR radiation.

400 mL of blood volume sample was incubated and thermally stabilized at 37 °C for 30 min in a polypropylene container prior to extracorporeal circulation. It was constantly mixed using a magnetic stirrer (ES 21H, WIGO, Pruszków, Poland) and a magnetic bar with a rotational speed equal to 250 rpm. Blood was irradiated for 15 min with an irradiance of 5.2 mW/cm<sup>2</sup> (Table 1). At the beginning of each experiment, the irradiance value on the sample's surface was measured with an Optel radiometer and adjusted according to the desired NIR dose. The control sample was subjected to the same activities as the irradiated samples except for exposure to NIR radiation. As a source of NIR, a combination of halogen reflector (MR11+C GU4 30°, 20 W, 12 V, ANS Lighting, Warsaw, Poland) and a polychromatic bandpass NIR filter (NIR IFG 098, Schneider-Kreuznach, Bad Kreuznach, Germany)

that transmits photons in a wavelength range of 750–1100 nm was used. Temperature stability measurements of the blood samples were performed after the construction phase using an electronic digital thermometer inserted into the transfer container concluding that NIR PBM did not change blood temperature. Previously, we used similar setup for the pulse NIR blood PBM [29].

**Table 1.** Photobiomodulation parameters.

Wavelength Range [nm]	750–1100; Noncoherent and Polychromatic Light
Irradiance (mW/cm <sup>2</sup> )	5.2
Power (mW)	146
Continuous-wave mode	No
Pulse sequence	Not applicable
No of pulses	1
Irradiation time (sec)	900
Spot area (cm <sup>2</sup> )	28
Energy (J)	131.4
Fluence (J/cm <sup>2</sup> )	4.7

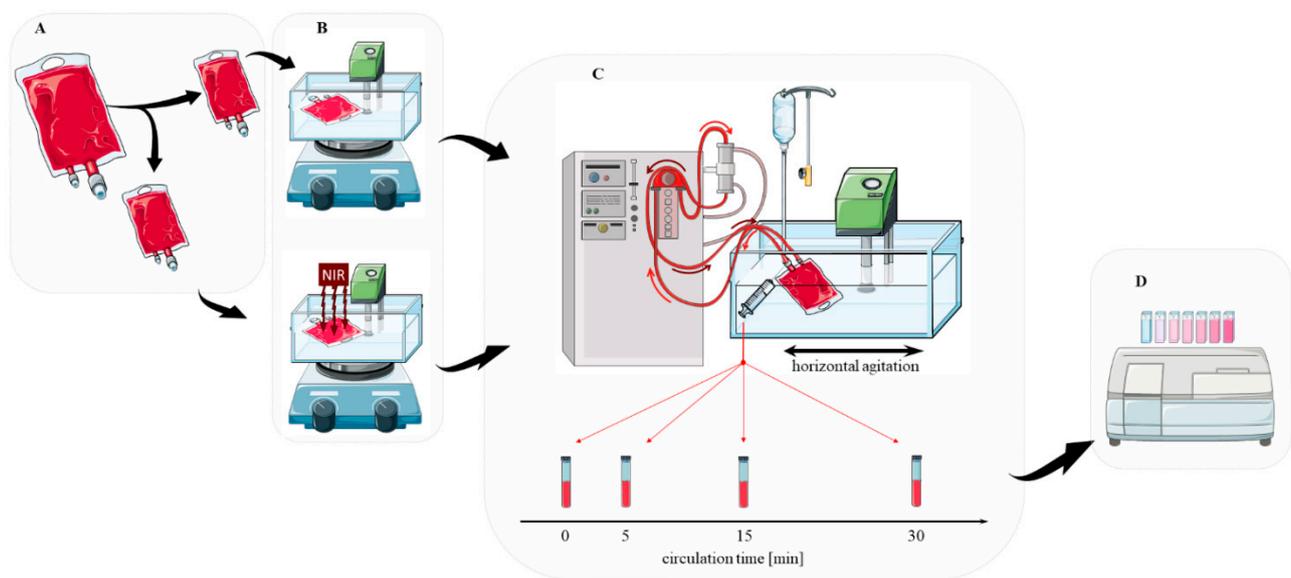
## 2.2. Experimental Setup

The most important element of the experimental setup of HD was the Fresenius 4008 HD apparatus (so-called “artificial kidney”) Fresenius 4008 (Fresenius Medical Care, Bad Homburg, Germany), which is a device designed to perform dialysis in dialysis departments and clinics, intensive care units, and at home. It allows the performing of dialysis procedures without the use of additional equipment. It ensures and supervises the circulation of dialysis fluid and extracorporeal blood circulation. The device controlled two circuits: blood and dialysate. The hydraulic system of the blood circulation consisted of the following elements: polyamide dialyzer (Polyflux 6 L, Gambro, Lund, Sweden) whose effective membrane area was equal to 1.4 m<sup>2</sup>, blood compartment volume 115 mL, and fiber inner diameter 250 μm; bloodline set (AV123, Gambro, Lund, Sweden), blood transfer container (Macopharma, Tourcoing, France), syringes and three-way stopcocks. The dialysate circuit consisted of concentrates A and B (257 MNT and 200 MTN, respectively, Neubrandenburg), a distilled water reservoir with a pump (08 024, Eheim, Deizisau, Germany). The mixing of concentrates was an automatic process performed by the HD apparatus. The resulting dialysis fluid was a multielectrolyte solution with a concentration similar to the plasma composition. In addition, an important element of the experimental setup was a horizontal stirrer (Helmer, Noblesville, IN, USA) that ensured the mixing of blood in a transfer container and prevented the sedimentation process of blood cells during circulation; thermostat was used to heat the blood to a temperature of 37 °C before and during extracorporeal circulation, and NIR radiator (Figure 1). The extracorporeal circulation using an HD apparatus was run alternately, once starting with a control (CTR) sample and the next time with an NIR sample. There were 13 independent experiments carried out. Each experiment consisted of CTR and NIR HD circulation.

## 2.3. Experimental Protocol

Before each experiment, the HD machine was subjected to a rinsing procedure (the “Rinse” program). At the same time, after its completion the disinfection process was carried out (the “Disinfection” program, Puristeril 340 liquid<sup>®</sup>, Fresenius, Bad Homburg, Germany). Then the blood circulation was prepared, flushed before introducing blood into it and filled with phosphate-buffered saline (PBS) solution. During the transfer of blood to the blood transfer container, which was part of the extracorporeal circulatory system, a set for blood and blood derivative transfusion with a filter with a mesh size of 200 μm was used. Blood was forced into the system while pushing part of the PBS. After filling the drains, the blood circulated at 250 mL/min flow for 30 min. A peristaltic pump

drove the flow. Blood flowed through the arterial line into the capillary dialyzer. Then, it returned through venous lines to the blood transfer container (the reservoir). The blood reservoir was placed on a horizontal stirrer to prevent the sedimentation of blood cells. The transfer container had two luer-lock connections. The first was connected to a system of two three-way stopcocks enabling blood infusion into the system and collecting blood samples. Another was connected to a three-way stopcock, allowing the introduction of infusion fluids into the transfer container. Due to the ultrafiltration process during HD treatment, saline was added to the circulating blood according to the indications of the ultrafiltration counter in the HD machine. It was essential to maintain appropriate time intervals and precision during this procedure to avoid falsifying the results by excessive dilution or blood concentration. These processes were directly controlled by measuring hematocrit (HCT) and the initial and final blood volume. The crucial parameters of the HD session were as follows: dialysate conductivity 14 mS/cm, dialysate flow rate 500 mL/min, dialysate temperature 37 °C, ultrafiltration 100 mL/h, transmembrane pressure—100 mmHg.



**Figure 1.** The concept of an experimental procedure. The blood was divided into two identical parts (A) followed thermal stabilization at 37 °C with or without exposition to NIR (B). CTR or NIR irradiated blood was introduced into the extracorporeal circuit of the HD machine and circulated for 30 min (C). Blood samples were taken at different time points for biochemical and biophysical analyses (D). Note: This figure contains graphic elements modified from Servier Medical Art (<http://smart.servier.com/>), accessed on 22 March 2022), licensed under a Creative Common Attribution 3.0 Generic License.

Blood samples for analysis were taken before circulation and after 5, 15, and 30 min of HD. The following parameters were determined: HCT, fHb, osmotic fragility, and oxidative stress (thiobarbituric acid reactive substances (TBARS) concentration).

#### 2.4. Cell-Free Hemoglobin Measurement

fHb measurements were performed as a hemolysis rate during HD treatment. Cell-free supernatant was obtained by centrifuging blood samples for 10 min at 1750× g. Each sample was measured in triplicate. The concentration of fHb was quantified spectrophotometrically (Nicolet Evolution 60, Thermo Scientific, Waltham, MA, USA) in each sample by a colorimetric assay using Drabkin's reagent (Aqua-Med., Łódź, Poland) at 540 nm.

#### 2.5. Hemolysis Curve and Determination of Osmotic Fragility

The osmotic fragility curve was performed as previously described [34]. Briefly, RBC were isolated by centrifugation at 1750× g for 6 min at 25 °C, washed three times in PBS of

pH = 7.4 and diluted in PBS to obtain 20% HCT. The RBC samples were placed in a series of tubes containing different concentrations of sodium chloride solution from isotonic to low ionic strength close to that of distilled water (0–145 mM NaCl buffered by 10 mM phosphate buffer, pH = 7.4). Thirty minutes after incubating RBCs in sodium chloride solution, the suspension was centrifuged at  $1750\times g$  for 10 min. The supernatant was examined spectrophotometrically (Nicolet Evolution 60, Thermo Scientific, Waltham, MA, USA). The amount of Hb released, proportional to the number of lysed cells, was estimated by colorimetric analysis at 540 nm. The distribution of the osmotic properties of blood cell populations in the studied NaCl concentration range was obtained by determining the first derivative of the hemolysis curve.

### 2.6. Hematocrit

The HCT measurement was performed by loading blood into a heparinized capillary (MEDLAB, Poland) with a length of 75 mm and a volume of 37  $\mu\text{L}$  followed by capillary centrifugation in a HCT centrifuge (MPW Med. Instruments 250 e, Warsaw, Poland) for 3 min at  $12,167\times g$ . The HCT value was estimated using a HCT reader apparatus (MPW Med. Instruments 16135, Warsaw, Poland).

### 2.7. Peroxidation of Red Blood Cell Membrane Lipids

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the TBARS concentration (Heath and Packer 1968) with little modification described in detail elsewhere [35]. Trichloroacetic acid (TCA, Chempur, Piekary Śląskie, Poland; 15% (wt/vol) TCA in 0.25 M HCl) and thiobarbituric acid (TBA, AppliChem, Darmstadt, Germany; 0.37% (wt/vol) TBA in 0.25 M HCl) were used. Equal volumes of RBC suspension, TBA, and TCA were mixed with 2% 2,6-Di-tert-butyl-4-methylphenol (BHT) (Fluka, Charlotte, NC, USA) in ethanol. The samples enclosed in small glass tubes were heated at 100 °C for 15 min, then cooled and centrifuged for 10 min at  $1750\times g$ . The absorbance of the supernatant was measured spectrophotometrically at 535 nm and corrected for nonspecific turbidity by subtracting the absorbance of the same sample at 600 nm. The content of TBARS was calculated using the extinction coefficient ( $155\text{ mM}^{-1}\text{ cm}^{-1}$ ).

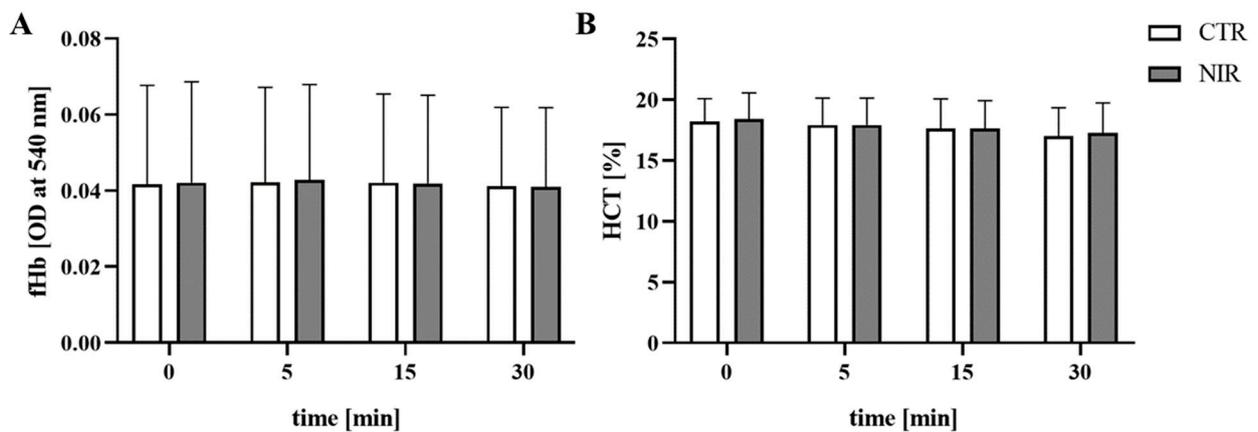
### 2.8. Statistical Analysis

Calculations were performed using the Statistica statistical package 13.3 (StatSoft Inc., Tulsa, OK, USA). Kolmogorov–Smirnov and Shapiro–Wilk tests tested the normal distribution hypothesis. A two-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed to determine significant differences between CTR and NIR treated samples at different time points of the experiment.

## 3. Results

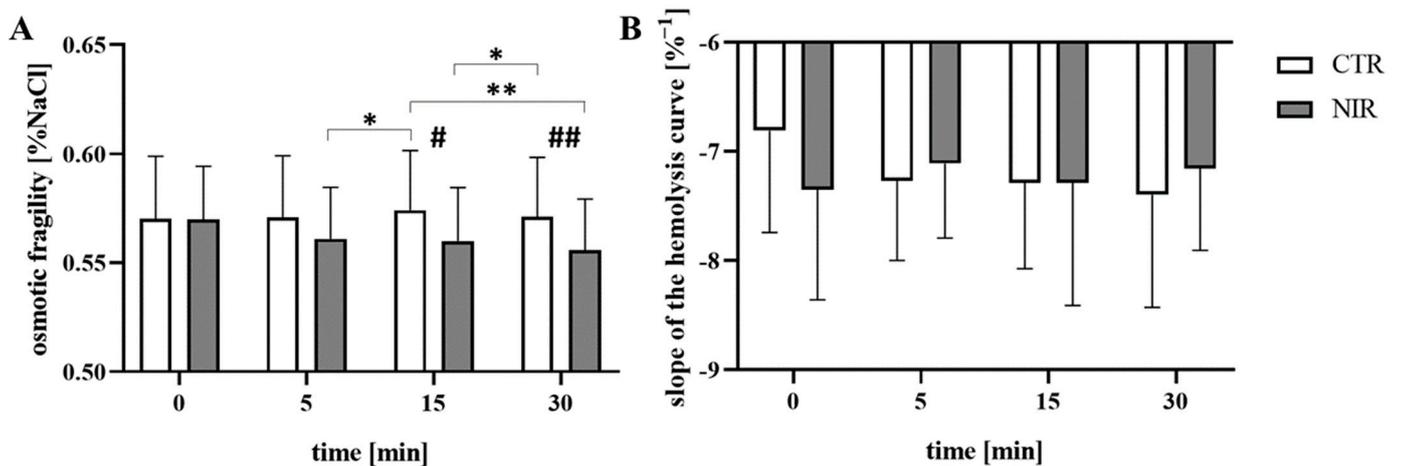
The evaluation of the antioxidative effects of NIR blood PBM during HD in vitro treatment consisted of assessment of the integrity of the RBC membrane and their osmotic properties (measurements of fHb concentration and the hemolysis curve respectively), as well as determination of the oxidative burst level (TBARS measurement) as the indicator of neutrophils activation and indirectly platelets due to contact with the dialysis membrane.

In vitro HD treatment did not result in an increased disruption of the RBC membrane and Hb outflow from cells to plasma (Figure 2A). The hemolysis level for the CTR and NIR samples maintained a constant value throughout the extracorporeal circulation. Similarly, no excessive concentration or dilution of blood was observed during HD treatment, which could be caused by an imbalance in the compensation process of fluids lost due to ultrafiltration (Figure 2B).



**Figure 2.** Changes in OD at 540 nm (ordinate) represent the results obtained from the fHb spectrophotometric assay that indicated RBC breakdown (A) and HCT (B) with time of blood circulation during HD in vitro treatment for CTR and NIR irradiated blood samples. Results are presented as mean and SD. The number of independent experiments  $n = 13$  for both groups. Statistical analysis did not show any differences between individual points, indicating that there was no breakdown of RBCs during the procedure. There was no dilution or blood concentration that may be caused by an imbalance of the process of compensating extracellular fluids lost as a result of ultrafiltration.

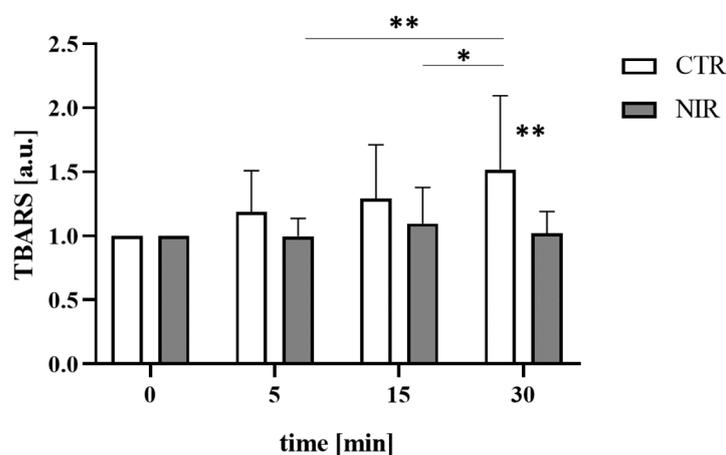
HD also did not induce significant changes in the osmotic fragility of the RBC in the CTR group, where values remained relatively stable throughout the whole circulation time. In turn, the use of NIR blood PBM resulted in a gradual reduction in osmotic fragility along with the duration of HD (Figure 3). Significant differences between CTR and NIR samples were observed in the 15 and 30 min ( $p < 0.5$  and  $p < 0.01$  vs. CTR, respectively) of the extracorporeal circulation. Concomitantly, there were no significant changes in the slope of the hemolysis curve during the experiment, indicating similar distributions of osmotic cell properties within the entire cell populations in both groups.



**Figure 3.** Changes in the osmotic fragility of RBCs (A) and the slope of the hemolysis curve (B) during in vitro HD. Pairs of variables that differed statistically from each other at the same time were denoted on the graphs with #  $p < 0.05$ , ##  $p < 0.01$ , while asterisks were used to mark other significantly different variables, \*  $p < 0.05$ ; \*\*  $p < 0.01$ . Results are presented as mean and SD. The number of independent experiments  $n = 13$  for both groups.

HD-induced oxidative stress was measured as the peroxidation of RBC membrane lipids at different experiment stages. In the CTR group, after 15 and 30 min of circulation, the concentration of TBARS increased by 30% and 50% of the starting value, respectively, while in the case of NIR PBM blood sample the increase was only 10% and 2%, respectively

(ns, and  $p < 0.01$  relative to the CTR group) (Figure 4). The action of NIR radiation, in this case, was extremely influential in limiting the generation of ROS.



**Figure 4.** Changes in lipid peroxidation of the RBC membrane during HD in vitro. Results are presented as mean and SD. The number of independent experiments measurements  $n = 13$  for both groups. Pairs of variables that differed statistically were denoted on the chart with the following symbols: \*  $p < 0.05$ , \*\*  $p < 0.01$ .

#### 4. Discussion

A common consequence of CRF, especially in patients receiving HD, is anemia attributed to inhibition of erythropoietin (a hormone responsible for reduced bone marrow activity), malnutrition, and reduced RBC life span in circulation [36]. Activation of the coagulation system during HD alone can lead to patient blood loss estimated at 300–750 mL per year [37], so it is clear that in CRF patients treated with HD, any lost blood volume matters. Another critical aspect we need to understand that occurs in these patients are cardiovascular complications, which are the leading cause of increased mortality [38]. The risk factor in this case is a chronic inflammatory process that results in increased oxidative stress [39] causing chronic deficiencies in the antioxidant system [39–42]. Peroxidation of membrane lipids of uremic patients accompanied by MDA production [36,39,41,43] reduces the deformability of RBC [36,43–45]. Uremia inhibits the activity of the  $\text{Na}^+/\text{K}^+$  pump [46], which, in combination with the increased influx of  $\text{Ca}^{2+}$  ions into cells due to elevated levels of parathyroid hormone (the effect of concomitant secondary hyperparathyroidism) [47,48], contributes directly to increasing the osmotic fragility of RBCs [36,42,45,49,50].

In this context, the method we have developed to protect blood cells in the extracorporeal circulation and the results obtained during in vitro HD are encouraging.

The main component of the HD blood circulation set-up activating the coagulation system is the dialyzer [51]. The degree of activation depends on the material from which the dialysis membrane is made [52,53]. As a measure of the biocompatibility of the dialysis membrane, oxidative stress can be used.

Herein, for the first time, we present the applicability of PBM of blood with polychromatic light in the NIR range to limit oxidative stress during in vitro HD treatment. In our in vitro model, the peroxidation of the membrane lipids of the RBCs gradually increased along with the extracorporeal circulation time. However, when blood was irradiated with NIR, the TBARS concentration remained constant throughout the experiment. Bearing in mind the processes taking place during contact of blood with biomaterials, the observed effect may result from inhibition of platelets that have not been activated on the dialysis membrane, inhibition of neutrophil activation leading to ROS formation, or modulation of the RBCs antioxidant system consisting of enzymatic antioxidants, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and peroxiredoxin-2 (Prdx2).

In our *in vitro* HD treatment model, we did not observe a direct effect of oxidative stress on osmotic fragility due to a gradual increase in TBARS concentration. This was not accompanied by a shift of RBCs osmotic properties in the CTR group. There was a gradual reduction in osmotic fragility in PBM blood. The explanation for this phenomenon may be the imposition of an additional process related strictly to HD. This can be attributed to the dialysis process, in which there are changes in the water-electrolyte balance of plasma. The important parameter to understand is the difference in the concentration of electrolytes in the extracellular fluid from the concentration of electrolytes in the dialysis fluid. Therefore, during the HD procedure, the composition of electrolytes in plasma may have changed, resulting in changes in the osmotic gradient between the extracellular and intracellular fluids, as a consequence of water migration from the cell to the environment (reduction in cell volume, e.g., in the NIR sample, not observed in the CTR sample due to, e.g., impaired cell regulatory mechanisms under the influence of oxidative stress). Nevertheless, changes in both osmotic fragility and oxidative stress prove the effective action of NIR radiation during HD. Exposed RBCs are less susceptible to membrane damage.

As mentioned above, NIR PBM could influence several processes translating to ROS formation during HD. On the one hand, platelets and leukocytes are activated during blood contact with the dialyzer membrane, so their inhibition could lead to reduced oxidative stress. On the other hand, RBCs possess high-capacity redox systems to provide protection against free radicals [54]. Previous studies with other cell types showed the effectiveness of red to NIR PBM in stimulating the activity of SOD and CAT [55,56]. This study was of application nature but based on the available literature, an additional concept of the NIR PBM triggered antioxidant mechanism of RBC protection can be hypothesized.

As we showed before, PBM with NIR radiation is a photochemical process where water plays a key role as the primary acceptor [57–62]. The absorption of NIR radiation by OH, NH groups causes overtone stretching vibrations, weakening hydrogen bonds created by them and altering the bound water structure [63–65]. As a result, the polarity of the environment decreases, facilitating structural changes of proteins and, changes within the charged groups. Therefore, the first stage of NIR action is a change in the water structure (i.e., the degree of hydration associated with biological structures).

Our previous research also confirmed that NIR PBM induces structural changes in Hb by modifying both the N-terminal and cysteine residues [66]. Furthermore, a dose-dependent reduction of functionally inactive met-Hb concentration [28] and an increase in deoxy-Hb [26,28] are observed. These changes are accompanied by intracellular acidification and modifications of band 3 proteins [28], which correlate well with variability of membrane fluidity [25], minimum value of zeta potential [26] and reduced membrane polarity [28,66].

Band 3, the anion transporter, is an integral membrane protein that anchors the plasma membrane to the spectrin-based membrane skeleton in RBC. It also plays a role in the assembly of glycolytic enzymes to regulate the metabolism of RBC and is able to recruit proteins that can prevent membrane oxidation. Deoxygenation leads to the phosphorylation of tyrosine in the band 3 protein, which causes a stronger network in the RBC cytoskeleton that can contribute to greater membrane mechanical resistance [67], which in turn, could explain the mechanism of RBC membrane stabilization and increased resistance to osmotic stress as the effect of NIR PBM.

Regardless of other antioxidant mechanisms in RBCs, lowering intracellular pH ( $pH_i$ ) induces the formation of active forms of a very potent antioxidative enzyme Prdx2. Human RBC Prdx2 is a hydrogen peroxide ( $H_2O_2$ ) scavenger that exists in the cytoplasm as dimers and decamers in dynamic equilibrium controlled by  $pH_i$ . A slight decrease in pH from the neutral value causes a strong shift of the equilibrium towards the formation of decamers [68]. The decameric form is in the hydrophobic area of the RBC membrane from the inside of the cell. The high abundance of Prdx2 (the third most abundant protein in RBC cytosol) shows that these proteins are essential players in peroxide detoxification in RBC. Prdx2 was found to be oxidized by endogenously generated  $H_2O_2$ , which was derived mainly from

Hb auto-oxidation. Although cellular location [69], Prdx2 is considered cytosolic and can also be linked to the RBC membrane, where it could be involved in defense mechanisms against lipid peroxidation. Prdx2 binds to the N-terminal cytoplasmic domain of band 3 and that the first 11 amino acids of this domain are crucial for Prdx2 membrane association in intact RBCs. The interaction of Prdx2 with band 3 experienced a conformational change up-on binding to band 3 without losing its peroxidase activity [70]. Our concept shows that despite other antioxidative enzymes, Prdx2 should also be included in further research on the mechanism of NIR blood PBM.

It is difficult to translate the results of this study directly into the blood of patients suffering from CRF since the differences in biochemical composition are too large. Furthermore, our study's possible limitation is its in vitro nature, which means that it does not provide detailed feedback on how PBM cells interact with other cells and tissues in the circulation and particularly in hemostatic conditions. However, our study is the first of its kind, providing a solid framework for further research in this field. Using freshly donated human whole blood units could deliver relevant insight for a better understanding of the interaction of NIR PBM blood with the dialyzer membrane, including dose-dependence analysis and the concept of light delivery to the extracorporeal circuit. The benefits of implementing NIR blood PBM appear to be multifaceted, including, on the one hand, a reduction in RBC clearance from the circulatory system, and therefore a better oxygenation of tissues, by reducing inflammation, ending with a decrease in platelet activation, leading to a new concept of anticoagulation management in HD patients. As stated above, animal studies that incorporate extracorporeal blood NIR PBM during cardiopulmonary bypass to diminish blood destruction showed cytoprotective effects without side effects. This is another argument for further research leading to the clinical application of the developed method during HD.

## 5. Conclusions

This work, for the first time examines NIR as a potential inhibitor for ROS generation during HD in vitro. Our results revealed that blood PBM with NIR radiation is an effective factor in reducing oxidative stress during in vitro HD treatment, and it contributes to a milder disturbance of the membrane osmotic properties of RBC.

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