



Research Article

# Protective Effects of Osthole against Free Radical-Induced Hemolysis of Erythrocytes

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## Abstract

**Background:** Osthole, one of the most active components of *Cnidium monnieri*, has different pharmacological and biological effects such as boosting the immune system, reducing rheumatoid pain, hepatoprotective, and inhibitory effect on osteoporosis. Furthermore, it showed anti-inflammatory, anti-cancer, and antioxidant properties. However, there is little information about the antioxidant effects of osthole using cell-based assays. In the current work, we used *in vitro* model of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis of erythrocytes to investigate the protective effects of osthole against oxidative damage of biological membranes.

**Methods:** Erythrocytes were challenged with 2, 2'-azobis (2-aminopropane) dihydrochloride (AAPH) as a model oxidant in the presence and absence of osthole. The protective effects of osthole on lipid peroxidation, protein carbonyl oxidation, glutathione (GSH) content of erythrocytes were evaluated and compared with control samples.

**Results:** It was found that osthole has protective effects on erythrocyte hemolysis induced by AAPH at different concentrations in a time-dependent manner. Osthole also suppressed lipid and protein oxidation as well as reductions in GSH content in a concentration and time-dependent manner.

**Conclusion:** Osthole showed protective effects against free radical-induced hemolysis in rat erythrocytes. Therefore, it can be considered as a supplement for the prevention or treatment of a variety of human health problems associated with oxidative stress. However, further investigations are required to illustrate other possible impacts of osthole on cells.

## Introduction

Oxidative stress is accountable for various pathological conditions known to affect several tissues and organs, thus being one of the most important and serious harms to human health.<sup>1,2</sup> Imbalanced conditions between the production of reactive species and antioxidant response lead to oxidative stress and thus, proteins and lipid peroxidation, DNA strand breakage, and impairment of membrane ion transport systems and enzymes.<sup>3</sup>

Due to the high O<sub>2</sub> pressure in arterial blood and their excessive heme iron content, Reactive Oxygen Species (ROS) are constantly produced in the erythrocytes. Hemoglobin (Hb) that is an oxygen carrier protein in erythrocytes is a source of ROS because it undergoes autoxidation to generate radicals of oxygen.<sup>4</sup>

Due to the presence of hemoglobin (Hb), polyunsaturated

fatty acids, and oxygen, erythrocytes may be prone to oxidative damage.<sup>5</sup> The exposure of erythrocytes to free radicals results in cell membrane damage (lipid peroxidation), oxidation of sulfhydryl group and protein cross-linking, changes in cellular morphology, subsequently lead to membrane disruption and hemolysis.<sup>6,7</sup> These phenomena occur in sickle cell anemia, thalassemia, dementia, glucose-6phosphate dehydrogenase (G<sub>6</sub>PDH) deficiency, and other kinds of hemoglobinopathies.<sup>8-11</sup> Erythrocytes have strong antioxidant activity including enzymatic and nonenzymatic mechanisms that together efficiently act to change ROS species into less reactive intermediate types.<sup>12</sup> However, their defense capability is limited because mature erythrocytes lack the *de novo* synthesis of antioxidative enzymes.<sup>13</sup> Thus, antioxidant

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agents can help prevent and treatment of many oxidative-mediated erythrocyte damages.

Osthole (7-methoxy-8-isopentenoxycoumarin) is a natural coumarin phytochemical that possess a broad range of pharmacological functions, including anti-apoptotic, anti-cancer, anti-inflammatory, antioxidative, and cardiovascular protective effects.<sup>14-17</sup> It also exerts an inhibitory effect on NF- $\kappa$ B pathway and ROS release in macrophages stimulated by LPS.<sup>18</sup> Besides, osthole has been shown to suppress NF- $\kappa$ B and p38 mitogen-activated protein kinase pathway, and diminish the malondialdehyde (MDA) concentration in FL83B cells that were treated with H<sub>2</sub>O<sub>2</sub>.<sup>15</sup> Osthole also showed antioxidant and anti-inflammatory activity in dinitrobenzene sulfonic acid induced-colitis in rats by reducing pro-inflammatory cytokines, myeloperoxidase (MPO), and MDA.<sup>19</sup> Zou *et al.* found that osthole can reduce oxidative stress caused by tamoxifen in acute liver injury in mice through the reduction in GSH, MDA, and H<sub>2</sub>O<sub>2</sub>. It also upregulated the expression of antioxidant genes such as superoxide dismutase, G<sub>6</sub>PDH, glutamate-cysteine ligase catalytic subunit, and glutathione peroxidase, while suppressed the expression of pro-oxidant genes such as NADPH oxidase and acyl-CoA oxidase 1.<sup>20</sup>

Although several studies on antioxidant activity of osthole already reported, there is no evidence available about the antioxidant effects of this compound on erythrocytes. In the present study, we used 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) as a generator of peroxy radical<sup>21,22</sup> and for the first time determined the protective effect of osthole against oxidative damage to rat erythrocyte membranes by evaluating hemolysis, protein and lipid peroxidation, and GSH content.

## Materials and Methods

### Chemicals

Trichloroacetic acid, 2-thiobarbituric acid (TBA), and dinitrophenylhydrazine (DNPH) were obtained from Merck (Germany). The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): AAPH, GSH, malondialdehyde tetrabutylammonium salt (MDA), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), Guanidine hydrochloride. Osthole was obtained from Golexir pars co., Iran.

### Preparation of erythrocytes

Twenty mature, male Wistar rats weighing 250-300 g, were used in this experiment. Rats were anesthetized with diethyl ether and direct punctation of the heart was used to obtain fresh blood. The blood samples were collected in heparinized tubes. All animal experiments were performed in compliance with the authorization of the Ethics Committee for Animal Experiments of Mashhad University of Medical Sciences. The samples were centrifuged at 2500 rpm for 20 min at 4°C in a refrigerated centrifuge (Sigma 3-30K; Sigma Co., USA) to separate erythrocytes from plasma and buffy coat. Then, samples

were washed twice with normal saline (0.9% NaCl) and once with phosphate buffer saline (PBS, pH 7.4).

Erythrocyte ghosts were prepared by following protocol. In order to lysis the erythrocytes, cold hypotonic PBS was added to erythrocyte solution (30 to 40 times the volume of erythrocytes) and incubated at room temperature for 1 to 3 days. Then, samples were centrifuged at 12000 rpm for 20 min at 4°C. The supernatants were discarded and the pellets were washed with normal saline for several times until the pellet became completely creamy. Finally, erythrocyte suspension (hematocrit 10%) was prepared in the same buffer and stored at 4°C. This solution used within 6 h of sample preparation.<sup>21-23</sup>

### Hemolysis assay

To determine the antioxidant activity of osthole on rat erythrocytes, AAPH was used to form peroxy radicals and induce oxidation in erythrocytes.<sup>24</sup> Erythrocyte suspensions at 10% hematocrit were preincubated at 37°C for 10 min and subsequently mixed with 200  $\mu$ l of 25 mM AAPH in PBS (pH 7.4). Then, 80  $\mu$ l of different concentrations of osthole (25, 50, 100, 200, 400  $\mu$ M) were added to the tubes. These reaction mixtures were incubated at 37°C for 6 h while shaken gently. Tubes containing erythrocyte suspended in PBS alone, erythrocytes mixed with AAPH alone, and erythrocytes mixed with AAPH and 100  $\mu$ M vit C were used as controls. At the indicated time points, a volume of 0.1 ml of the reaction mixture was removed and diluted with 0.9 ml PBS and centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant (A) was measured at 540 nm and compared with the reference value (B). Determining the reference value was performed by utilizing the same volume of the reaction mixture in hypotonic PBS (5 mM, pH 7.4; 100% hemolysis). The degree of hemolysis was calculated from the ratio of the readings (A/B)  $\times$  100.

### Evaluation of lipid peroxidation in erythrocytes

Thiobarbituric acid (TBA) assay was performed to assess lipid peroxidation with some modifications.<sup>25,26</sup> One ml of TBA reagent (0.375% (w/v) TBA, 0.25 M HCl, 15% (w/v) TCA) was mixed with 0.5 ml reaction mixture and incubated at 95 °C for 1 h. After cooling and centrifugation at 1000 rpm for 10 min, the reaction mixture at 1000 rpm for 10 min, the absorbance of the supernatant was measured at 532 nm. To quantify the amount of MDA, a standard curve was constructed. For this purpose, different concentrations of MDA (5, 10, 20, 30, and 50  $\mu$ M) were prepared. Then, 2 ml of TBA reagent was added to 1 ml of each sample and incubated at 95°C for 1 h. After the indicated time, the absorbance of the samples was measured at 532 nm and the standard curve was plotted and presented as nmol/mg Hb. In order to estimate the Hb content by colorimetric method, another aliquot of the lysate was also used. For determination of total Hb, standard cyanmethemoglobin method<sup>27</sup> using Drabkin's reagent was performed.

### Estimation of GSH content in erythrocytes

The levels of GSH in erythrocyte was measured according to the method reported by Yang *et al.*<sup>28</sup> At intervals of every 2 h, 2 ml of reaction mixtures containing 10% erythrocyte suspension mixed with 25 mM AAPH and treated with different concentrations of osthole, and were centrifuged. Then, 0.6 ml of cold hypotonic PBS was added to cell pellets to lyse the cells. Subsequently, 0.5 ml metaphosphoric acid solution containing 30 g NaCl, 0.2 g EDTA, 1.67 g metaphosphoric acid in 100 ml water was added to 0.5 ml of the erythrocyte lysate. After 5 min, centrifugation at 18000 rpm for 10 min was performed to precipitate the proteins and separate them from the remaining solution. Then, 0.45 ml of the solution was mixed with Na<sub>2</sub>HPO<sub>4</sub> (0.45 ml; 300 mM), and the absorbance was measured at 412 nm. After that, 100 µl DTNB solution containing 20 mg DTNB in 100 ml 1% sodium citrate was added to the sample and blank, and absorbance of samples was read at 412 nm. The GSH concentrations were expressed as mol/g Hb. In order to quantify the amount of GSH, a standard curve was prepared using 20-100 µM of GSH solution. Then, 0.45 ml of GSH solution was mixed with Na<sub>2</sub>HPO<sub>4</sub> (0.45 ml; 300 mM) and the absorbance was measured at 412 nm. Finally, 100 µl of DTNB solution was added to the samples and blank, and the absorbance of samples was read at 412 nm.

### Determination of protein oxidation in erythrocytes

The protein carbonyl of the erythrocyte membrane was measured according to the standard available methods.<sup>29,30</sup> Reaction mixtures (2 ml) were centrifuged and 0.6 ml of cold hypotonic buffer was added to the cell pellets to lyse the erythrocytes. Next, 0.1 ml of the cell lysate was collected into two tubes as test and control. Then, the test sample was mixed with 0.8 ml of 10 mM DNPH prepared in HCl (2.5 M) and control sample was mixed with 0.8 ml of 2.5 M HCl. The samples were incubated in the dark at room temperature for 1 h. To facilitate the reaction with proteins during the incubation, the tubes were occasionally shaken every 10 min. Then, both test and control tubes were mixed with 1 ml of 20% TCA and incubated on ice for 10 min.

After centrifugation of the tubes at 10,000 rpm for 10 min for protein precipitation, 1 ml of 10% TCA (w/v) was added to the protein pellets and the tubes left on ice for 5 min. Protein pellets were obtained by the centrifugation of the tubes at 10,000 rpm for 10 min. In order to remove lipid remnants and unreacted DNPH, the pellets were washed with ethanol: ethyl acetate solution (1:1, v/v) for three times. Finally, guanidine hydrochloride (6M) was added to the protein pellets and incubated at 37°C for 10 min. The centrifugation step was done to remove the insoluble materials. The carbonyl content of test samples was determined against the control samples by reading the absorbance of the supernatant at 370 nm. For standard solution, bovine serum albumin (BSA) was added to guanidine hydrochloride (6 M) and different concentrations of BSA were prepared (50-2000 µg/ml) and

the protein value in the supernatant was determined at 280 nm. An absorption coefficient ( $\epsilon$ ) of 22,000 M<sup>-1</sup> cm<sup>-1</sup> was utilized to calculate the carbonyl content, and the data were expressed in nmol/mg protein.

### Statistical analysis

The analysis of data for repeated measures (ANOVA) and Turkey-Kramer test were used for statistical analyses. Data were expressed as mean  $\pm$  SD. Statistical significance was defined as  $p < 0.001$ .

## Results

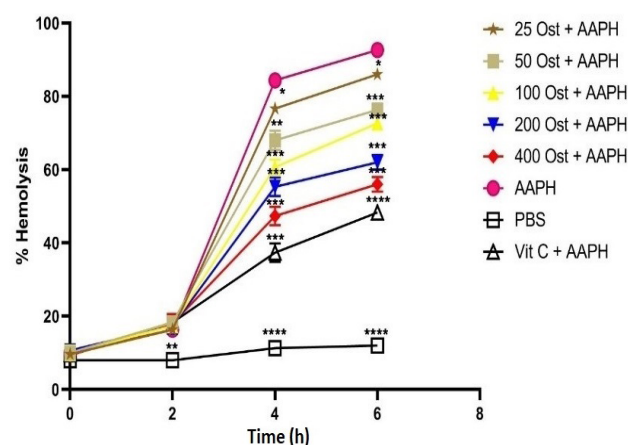
### Effect of osthole on AAPH-induced hemolysis in erythrocytes

Erythrocytes suspended (10%) in PBS alone and incubated at 37°C, showed little hemolysis within 6 h (less than 10%; negative control). When 25 mM AAPH was added to the erythrocyte suspension, oxidative stress resulted in time-dependent hemolysis of erythrocytes. Osthole inhibited AAPH-induced hemolysis of erythrocytes in a concentration- and time-dependent manner ( $p < 0.001$ ) as shown in Figure 1.

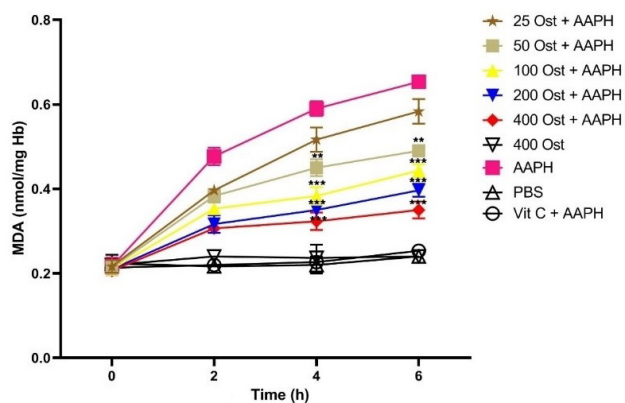
Higher concentrations of osthole (100 and 200 µM) showed more inhibitory effect on oxidative hemolysis. The simultaneous addition of osthole showed better protective effects in comparison to its delayed addition, by 2 h relative to AAPH (data not shown) (Figures 1-4). The positive control (100 µM vit C+AAPH) also showed anti-oxidant and anti-hemolysis activities as are shown in Figure 1.

### Effects of osthole on AAPH- induced lipid peroxidation

Incubation of erythrocyte suspensions with 25 mM AAPH considerably leads to an intensive process of lipid peroxidation compared to the respective control ( $p < 0.0001$ ). After the erythrocyte suspensions were mixed with AAPH, lipid peroxidation was observed in a time-dependent manner. As shown in Figure 2, osthole significantly suppressed the AAPH-induced MDA



**Figure 1.** Effect of different concentrations of osthole on AAPH-induced hemolysis in erythrocytes. Data are expressed as the mean  $\pm$  SD ( $n = 3$ ), \*\*\* $p < 0.001$  vs AAPH



**Figure 2.** Effect of osthole on AAPH-induced changes in MDA content of erythrocytes. Data are expressed as the mean±SD (n = 3), \*\*\*p < 0.001 vs AAPH.

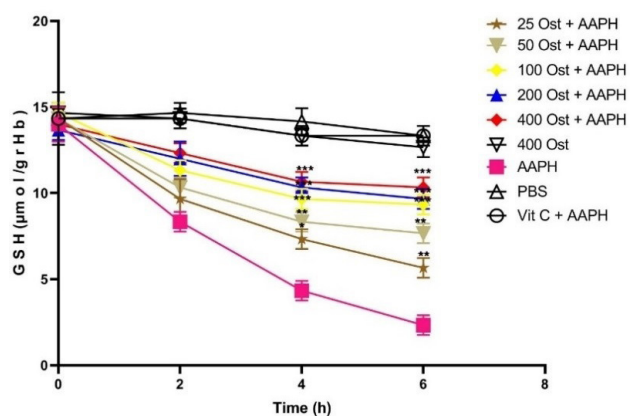
formation in a concentration-dependent manner ( $p < 0.0001$ ,  $p < 0.005$  for 50  $\mu\text{M}$  of osthole). Incubation of erythrocytes with osthole in the absence of AAPH, resulted in maintenance of MDA formation at a background level similar to that of controls.

#### Effects of osthole on AAPH- induced changes in GSH levels of erythrocytes

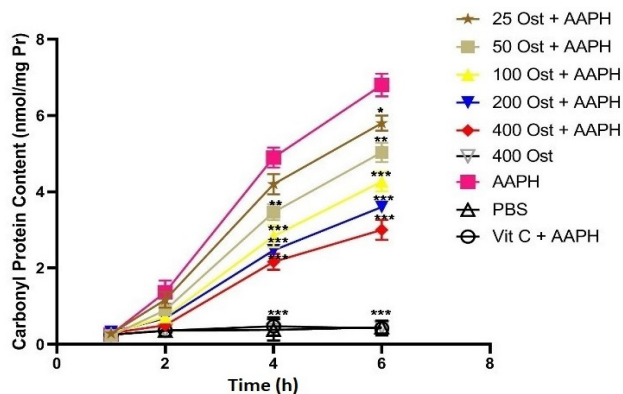
A considerable time-dependent decrease in the cytosolic GSH content of erythrocytes was observed when erythrocyte suspensions were incubated with 25 mM AAPH. After the addition of osthole, a remarkable suppression in AAPH- induced cytosolic GSH uptake was observed in a concentration- dependent manner ( $P < 0.001$ ) while, erythrocyte suspension incubated with osthole, in the absence of AAPH did not resemble significant alteration in the content of GSH after 6 h incubation that was similar to the AAPH- untreated samples (Figure 3).

#### Effects of osthole on AAPH- induced changes in protein carbonyl content of erythrocytes

The assessment of protein carbonyl is an indicator of protein oxidation in oxidant-treated erythrocytes.



**Figure 3.** Effect of osthole on AAPH-induced changes in GSH content of erythrocytes. Data are expressed as the mean±SD (n = 3), \*\*\*p < 0.001 vs AAPH.



**Figure 4.** Effect of osthole on AAPH-induced changes in protein carbonyl content of erythrocytes. Data are expressed as the mean±SD (n = 3), \*\*\*p < 0.005 vs AAPH.

Incubation of erythrocytes with osthole alone, did not show any changes in the protein carbonyl content, similar to that of AAPH- untreated samples. The addition of 25 mM AAPH, results in a remarkable increase in the amount of protein carbonyl in a time-dependent manner compared to respective control sample ( $p < 0.005$ ) (Figure 4). After the addition of osthole to AAPH- treated erythrocytes, a significant inhibition in the production of protein carbonyl was observed in a concentration- dependent manner ( $P < 0.005$ ) A considerable decrease in the inhibitory effect of osthole on the AAPH-induced lipid peroxidation, GSH depletion, and protein oxidation were observed after delayed addition of osthole and AAPH to erythrocytes (data not shown). However, the simultaneous addition of osthole relative to AAPH showed better results as are shown in Figure 4.

#### Discussion

Osthole has been shown to exert different pharmacological effects, including anti-inflammation, antioxidative, and anti-apoptosis in several studies.<sup>31,32</sup> Although, many studies have been carried out to investigate the antioxidant function of osthole in different diseases such as liver, arthritis, and Alzheimer's.<sup>33-35</sup> very little information is available regarding to its activity using cell-based assays. Hence, this study was designed to assess the efficacy of osthole as an inhibitor of AAPH- induced oxidative damage in normal rat erythrocytes.

In the circulation, erythrocytes are constantly subjected to both endogenous and exogenous sources of ROS that can affect their function and cellular integrity. Oxygen-rich environment and the presence of polyunsaturated fatty acids in the membrane of erythrocytes make them susceptible to lipid peroxidation, and also iron-rich Hb make erythrocytes prone to oxidative damages. Hb undergoes slow autoxidation, resulting in the production of methemoglobin (that can no longer carry oxygen) and superoxide that quickly form hydrogen peroxide.

Erythrocytes have potent antioxidant systems involving non-enzymatic antioxidants like GSH and vit C and enzymatic antioxidants such as glutathione peroxidase,

superoxide dismutase, catalase, and peroxiredoxin-2.<sup>36,37</sup> However, the lack of de novo synthesis of antioxidant in mature erythrocytes and imbalanced oxidant/antioxidant equilibrium in several erythrocyte-related diseases, highlight the importance of using antioxidant agents to prevent or decrease oxidative damage to erythrocyte membranes. Oxidative damage can affect proteins and lipids within the erythrocyte membrane and contributing to some hemoglobinopathies. Thus, providing models to evaluate erythrocytes hemolysis inhibition are suitable strategies to study cell membrane oxidative damage induced by free radicals.<sup>38,39</sup>

In this study, osthole showed an inhibitory effect on AAPH-induced oxidative hemolysis of erythrocytes in a concentration and time-dependent manner. The results showed that the highest concentration of osthole (400  $\mu\text{M}$ ) can reduce 40% of erythrocyte hemolysis although it was less compared to vit C as the positive control. Moreover, 400  $\mu\text{M}$  of Osthole was able to attenuate the morphological changes of erythrocytes caused by AAPH compared to vit C as the positive control (data not shown).

Lipid peroxidation of cell membranes is proposed as a common mechanism for disruption of cell membrane and cell death. Lipid peroxidation contribute to protein dislocation and subsequently losing the integrity of cell membrane.<sup>40</sup> In the presented results, osthole was able to inhibit the production of MDA by oxidized lipoproteins in a concentration-dependent manner. According to the results, osthole showed the most inhibitory effect on MDA formation within 4 and 6 h of incubation ( $P < 0.005$ ). Kong *et al.* reported that osthole can reduce MDA and increase SOD contents and therefore inhibited oxidative stress in the serums of rats. It also repressed the TLR4, and NF- $\kappa\text{B}$  expression and enhanced SIRT1 expression implying that osthole functions through SIRT1/TLR4/NF- $\kappa\text{B}$  signaling pathway.<sup>35</sup>

GSH is a substrate for glutathione S-transferase and glutathione peroxidase and is a widely available hydrophilic antioxidant in mammalian cells. Oxidative stress decreases the cellular amount of GSH, therefore increases lipid peroxidation which results in DNA damage.<sup>41-43</sup> We found that the treatment of erythrocytes with AAPH for 6 h resulted in a 90% reduction in the intracellular level of GSH that indicates the sensitivity of GSH to oxidative stress induced by APPH. Based on the results of the simultaneous addition of osthole and AAPH, it was found that 100, 200, and 400  $\mu\text{M}$  concentrations of osthole in simultaneous testing were able to significantly ( $P < 0.001$ ) prevent the AAPH-induced reduction of GSH content.

An early indicator of protein oxidation that contributes to the disruption of the cellular membrane is introducing carbonyl groups in proteins at some amino acid residues.<sup>44</sup> Our results indicated that osthole can protect the erythrocytes against protein oxidation caused by AAPH in a concentration-dependent manner.

## Conclusion

According to the results of this study, we reported for the first time that osthole can prevent APPH-induced hemolysis in erythrocytes by inhibiting lipid and protein oxidation as well as by reduction in GSH content. These findings suggest that osthole may have antioxidant activity and cytoprotective properties. Therefore, it can be taken into account for the treatment and prevention of many free radical-mediated diseases that lead to erythrocyte damage such as thalassemia, G<sub>6</sub>PDH deficiency, sickle cell anemia, etc. However, complete recognition and characterization of other possible mechanisms require further studies.

## Ethical Issues

The study protocol was approved by the Ethical Committee for Animal Experiments of Mashhad University of Medical Sciences (IR.MUMS.PHARMACY.REC.1397.080) and also followed the National Institutes of Health Guide for care and use of laboratory animals (NIH Publications "No. 8023, revised 1996").

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## Conflict of Interest

The authors claim that there is no conflict of interest.

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