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Hepatoprotective, DNA Damage Prevention and Antioxidant Potential of *Spirulina platensis* on CCl4-Induced Hepatotoxicity in Mice

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Abstract In the present study, we have evaluated the hepatoprotective and antioxidant effects of *Spirulina platensis* against CCl_4-induced hepatotoxicity in mice. Activities of liver marker enzymes; Alanine transaminase and Aspartate transaminasewere estimated, as well as lipid peroxidation and antioxidant status (glutathione peroxidase) were determined in liver homogenate. DNA damage in liver was also evaluated by means of Comet assay. CCl_4 induction (1 mg/kg b.wt) significantly increases the levels of liver marker enzymesand lipid peroxidation, and caused the depletion of antioxidant status. Treatment of *Spirulinaplatensis* (800mg/kg/b.wt) to CCl_4 challenged mice resulted in decreased liver marker enzymes activity, DNA damage and lipid peroxidation levels with increase in antioxidant status. Our study clearly demonstrates that *Spirulina platensis* shows hepatoprotective effect through its antioxidant activity on CCl_4-induced hepatotoxicity in mice.

Keywords: *blue green algae, lipid peroxidation, liver marker enzymes, mice, comet assay*

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1. Introduction

Chemotoxic effects of chemicals or drug have been reported onthe all body parts (organs) but some organs are more sensitive than the others and show higher toxicity. The liver is the largest internal organ in the body and played a vital role in the detoxification of harmful substances. It has regulatory effect on the many important metabolic functions and is responsible for maintaining homeostasis of the body [25]. The concentration of the free radical is normally very low inthe healthy organisms than the diseased person because theyhave capacity to neutralize, metabolize or subtract the toxiceffects by free radical scavengers. The induction of excessivefree radicals during metabolism may cause liver damage [13].

CCl_4 produces an experimental damage that histologically resembles viral hepatitis [34]. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes locatedin the intracellular structures [24]. The toxic metabolite CCl_4 radical is produced which is further converted to trichloromethyl peroxy radical by cytochrome P450 2E1 enzyme. This radical binds covalently to the macromolecules and causes peroxidative degradation of cellular membrane leading to the necrosis of hepatocytes [33]. Interestingly, The oxidative DNA damage of CCl_4 was evaluated by means of the comet assay, which is widely used in genotoxicity testing *in vitro* and also becoming an important tool for evaluating the genotoxic potential and mutagenicity of many chemicals and natural compounds *in vivo* where as it play important roles in the determination of DNA damage level [27].

Spirulina (Blue green algae) is a microscopic single cell alga which grows in fresh water and has a simple structure but a complex composition. It is a concentrated source of food containing nutraceutical, antioxidants, probiotics properties. Moreover, it is an important source of the blue photosynthetic pigmented protein C-phycocyanin, which has strong antioxidant and anti-inflammatory properties. Interestingly, *spirulina* is known for its wide ranging biological activities, like prevention of anemia because of high iron and vitamin contents [17], inhibition of herpes simplexinfection [12], reduction in HIV replication velocity [6], increased production of antibodies, prevention of proliferation of neoplastic cells [32], hypoglycemic [2,30] hypolipidemic [21] and antihypertensive properties in experimental animal and humans models [31], furthermore, it shows hepatoprotective properties through decreasing of the liver lipid profiles and lipoperoxidation products [1], antimutagenic, antiviral, immune enhancing, cardioprotective and anticancer properties [23]. It attracted attention due to its ability to

stimulate mineralabsorption by its effects on intestinal microflora, carbohydrates, polyunsaturated fatty acids, sterols [35] moreover the presence of some more vital elements like calcium, iron, zinc, magnesium, manganese and selenium was also reported [15]. Some evidence also suggests that *Spirulina* can act on bone metabolism but at present no experimental evidence areavailable on this interesting and important aspect [39]. Hence, an attempt has been made to investigate thechelating mechanism followed by *Spirulina* against the CCl_4 toxicity in Swiss Albino mice. *Spirulina* has a property of reducing heavy metals and nephrotoxic substances from the body [10]. The present investigation was designed to examine possible potentials of *Spirulina* against hepatic intoxication induced by CCl_4 in male mice in an attempt to understand its mechanism of action, which may pave the way for the possibility to use it for therapeutic application.

2. Materials and Methods

2.1. Animals

Fifty Swiss Albino mice weight between 29 to 33 gm were obtained Institute of Graduate Studies and Research (IGSR), University of Alexandria.during the experimental period these were kept in awell-ventilated animal house and under the control managerial and environmental conditions. These animals were divided in five groups of ten animals each. CCl_4 (1 ml/kg body weight) was administered to groups III and groups IV animals by subcutaneously injection which is well documented to induce acute hepatotoxicity in mice. All animals were made to fast 24 h before the experiment. The duration of the experiment was every other day for three successive weeks.

Group I: This group received normal saline (0.9%NaCl) served as negative control.

Group II: Animal in this group received subcutaneously olive oil (0.5 ml/kg body weight/day)

Group III: Each animal received subcutaneously CCl_4 (1 ml/kg body weight/day) diluted with olive oil (1:1) as a solvent for the CCl_4.

Group IV: Animals were pretreated with *Spirulina* (800 mg/kg body weight/ 0.5 ml drinking water) orally 30 min after the single injection of CCl_4 (1 ml/kg body weight/day).

Group V: *Spirulina platensis* group which received Spirulina (800 mg/kg body weight / 0.5 ml drinking water).

At the end of experimental period, mice were slightly anaesthetized by diethyl ether (Sigma Chem. Co., St Louis, Mo. U.S.A.) and liver was carefully excised from each mouse and immediately immersed in a saline solution (0.9% NaCl). Liver homogenates (10%) were prepared in 0.01M Tris-HCl buffers (pH 7.5). The homogenate were centrifuged at 4000 r.p.m for 15 min, and the resultant supernatants were frozen at -20°C for hepatic parametersassay.

2.2. Chemicals

All reagents were of the highest purity available. CCl_4 was purchased from Sigma-Aldrich (St. Louis, Mo, USA). *Spirulina* powder was kindly provided from Dr. Heba Saad at The National Institute of Oceanography and Fisheries (NIOF); Egypt.

2.3. Biochemical Assays

Liver samples were homogenized in phosphate buffer for the assessment of aspartate transaminase (AST) and alanine transaminase (ALT) calorimetrically using kits purchased from Bio- Diagnostic, Egypt. (CAT. NO. AS 1061 (45) and CAT. NO. AL 1031 (45), respectively).

2.4. Biomarkers of Oxidative Stress

Lipid peroxidation in liver was estimated calorimetrically by measuring Malondialdehyde (MDA) by the thiobarbituric acid assay procedure using kit purchased from Bio- Diagnostic, Egypt (CAT NO. MD 2529). Glutathione peroxidase (GPx) wasdetermined using kit purchased from Bio- Diagnostic, Egypt (CAT NO. GP 2524).

2.5. Evaluation of DNA Damage (Comet Assay)

Crushed samples were transferred to 1 ml ice-cold PBS (phosphate buffer saline, pH 7.9). This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was mixed with 600 µl of low melting agarose (0.8% in PBS), where 100 µl of this mixture was spread on the slides. The coated slides were immersed in lyses buffer (0.045 M TBE, tris borate EDTA pH 8.4, containing 2.5% SDS) for 15 min. The slides were then placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 1V/cm for 20 min and 100 mA. Finally, the slides were stained with ethidium bromide (EtBr) 20µg/ml at 4°C. The observation was the samples still humid and the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (With excitation filter 420-490 nm [issue 510 nm]). The comets tail lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBr-stained DNA using a 40x objective on a fluorescent microscope. Although any image analysis system may be suitable for the quantitation of single cell gel electrophoresis (SCGE) data, we use a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a charge-coupled device (CCD) camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample [28].

2.6. Statistical Analysis

Data were subjected to one-way analysis of variance applying SAS program [38] using general linear model GLM. Significant differences among treatment means were separated using Duncan's multiple range procedure [11]. The values are expressed as means ± S.E. for 5 rats in each group. *P-Values*< 0.05 were considered significant according to Snedecor & Cochran.

3. Results and Discussion

3.1. Biomarkers of Oxidative Stress

The liver is the most sensitive organ for peroxidative damage because it is rich in oxidizable substances. The improvement in the oxidative stress of liver cells and the consequently decrease in the antioxidant ability of the cells caused an aggressive cellular damage in those cells in which destruction of membranes occurred and enzymes were released into the blood stream. So**,** the present study revealed the hepatoprotective activity of *Spirulina* against well knownhepatotoxins produced by CCl_4. It is worth mentioning that the olive oil was added as a solvent to CCl_4, since it is considered as less harmful to the liver in acute CCl_4 poisoning than other oils. In this context, Group II in the designed animal model was treated with olive oil only to avoid any misleading results in the treatment effects of Spirulina whether being from it or from the olive oil added to the toxicant CCl_4 in Group IV.

In the assessment of liver damage by CCl_4, the determination of enzyme levels such as alanine transaminase (ALT) and aspartate transaminase (AST) is largely used. In the present study, the rise in the levels of ALT, AST in CCl_4 treated mice has been attributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm and are released into the circulation after cellular damage [37]. Chaung*et al*., [9] demonstrated that ALT enzyme is one of the indices of the degree of cell membrane damage whereas AST is one of the indicators of mitochondrial damage, since mitochondria contain 80% of the enzyme.

As shown in Table 1, activities of AST and ALT were markedly elevated in CCl_4 treated animal groups compared to control group, indicating liver injury. The activities of AST, ALT were increased after administration of CCl_4; this incidence indicates that liver damaged by CCl_4. The increased activities of hepatic enzymes; AST, ALT in CCl_4 treated animal groups may be attributed to cellular leakage and loss of functional integrity of cell membrane in liver. The present results are in agreement with the results of Abraham [3] and Pari & Suresh [29], The increased levels of serum enzyme such as AST and ALT indicated the increased permeability and damage or necrosis of hepatocytes Ibrahim et al [19] &Hurkadale et al [18] in which hepatic markers were elevated.

Table 1. Liver biochemical parameters under normal and experimental conditions

Biochemica parameter / Groups	AST (U/ml)	ALT (U/ml)	Glutathione peroxidase (U/mg. protein)	Malondaldehyde (MDA) level (nmol/mg protein)
Control	369±6.78 ^{c}	37.72±1.22 ^{b}	13.60±1.66 ^{a}	14.90±1.40 ^{c}
Olive Oil	395±14.97 ^{bc}	37.10±0.48 ^{b}	13.40±10.93 ^{a}	14.99±0.99 ^{c}
CCl4	521.80±41.17 ^{a}	47.02±3.55 ^{a}	8±0.71 ^{b}	29.11±1.52 ^{a}
Spirulina+ CCl4	437.75±18.23 ^{b}	40.60±0.76 ^{b}	11.60±0.93 ^{a}	19.97±1.27 ^{b}
Spirulina	368.40±0.93 ^{c}	37.25±0.62 ^{b}	13.20±0.80 ^{a}	14.68±0.38 ^{c}

The results are presented as means ± SE of five mice.Letters (a-c) mean within a column not sharing similar superscripts in each classification are significantly different (P ≤0.05).

Moreover, administration of *Spirulina* at 800 mg/kg body weight, significantly ($p<0.05$) lowered the elevation of enzymes induced by CCl_4 in relation to control group (Table 1). Bhat and Madyastha [7] reported that the presence of blue pigment phycocyanin in *Spirulina* reduced the hepatotoxicity caused by CCl_4 – induced free radicals. Reduction in the levels of liver enzymes induced by *Spirulina* attributed to the inhibition of reaction involved in the formation of reactive metabolites and its radical scavenging activity. The presence of β-carotene, enzyme superoxide dismutase, vitamins or selenium in *Spirulina* produced immunostimulant activities and protective effects against CCl_4 – induced liver damage [4]. Also, Sharoud showed that treatment with *Spirulina* significantly restored this enzyme activity to be approximately near the normal limits in most of the cases.

On the other hand, glutathione peroxidase (GPx) is another enzymatic biomarker for liver damge. Data (Table 2) revealed that there was significant decreases in GPx values, in the CCl_4 treated animals as compared with their corresponding control values.

Murugesh *et al*., [26] indicated that liver enzymes including the above mentioned enzymes; glutathione peroxidase are thought to be the fundamental antioxidant enzymes, for they are closely related to the direct elimination of reactive oxygen species. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. On the same trend, [36] conducted that *Spirulina* treatment was observed to exhibit hepatoprotective effect as demonstrated by enhanced activities of antioxidant enzymes including glutathione peroxidase.

Lipid peroxidation in liver was estimated calorimetrically by measuring Malondialdehyde (MDA) by the thiobarbituric acid assay procedure. Subcutaneous administration of CCl_4 (1mg/kg. body weight), to mice markedly increased the lipid peroxidation in the tissue of liver as indicated by the highly significant increase ($p< 0.0001$) in its in comparison with the control value in CCl_4 mice Table1, It is well documented that liver tissue contains a relatively high content of polyunsaturated fatty acids, which are sensitive to peroxidative damage. Moreover, MDA was elevated in hepatic tissues after administration of CCl_4 could be expected reduced GPx activity [14].

Mice treated with *Spirulina*reduced (MDA) in comparison with CCl_4 toxin group (Group 3) Table1. The administration of *Spirulina* biomass to CCl_4-treated mice altered the above changes by regulating the MDA level and restoring antioxidant enzymes. Most of the active constituents present in the *Spirulina*have been reported to

be a potent inhibitor of lipid peroxide formation, a scavenger of hydroxyl and superoxide radicals, and to increase the antioxidant enzymes [40]. Moreover C-phycocyanin present in Spirulina inhibited paracetamol-induced lipid peroxidation in mice liver cells Gini & Muraleedhara [15]. So some of the active constituents of *Spirulina*such as flavonoids, β-carotene and phycocyanin have been reported to possess strong antioxidant activity and provokes free radical scavenging enzyme system. The significant protection offered by C-phycocyanin demonstrated the radical scavenging activity and its inhibitory effect on lipid peroxidation chain reaction [2]. Similarly, a previous study evaluated the hepatic MDA level and revealed that it was increased in galactosamine-intoxicated mice, whereas a significant decrease in antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase) and total reduced glutathione was observed [36].

3.2. Evaluation of DNA Damage (Comet Assay)

Liver DNA damage was revealed by various comet assay parameters that were provided by the image analysis software including tail length, % of DNA in the tail and tail moment (Table 2 and Figure 1).

Table 2. Oxidative DNA damage in the liver cell bearing male mice in different groups

Sample	Tail length µm	% DNA	Tail moment unit
1	1.96± 0.7	0.398±0.1	0.673±0.04
2	1.28±0.5	0.91±0.4	1.356±.05
3	5.88±0.4*	2.967±0.6*	17.445±2.23*
4	2.22±0.5	2.441±0.5*	5.41902±1.57
5	2.21±0.6	1.016±0.4	2.924±1.48

The results are presented as means ± SE. Level of significance is at P<0.05.

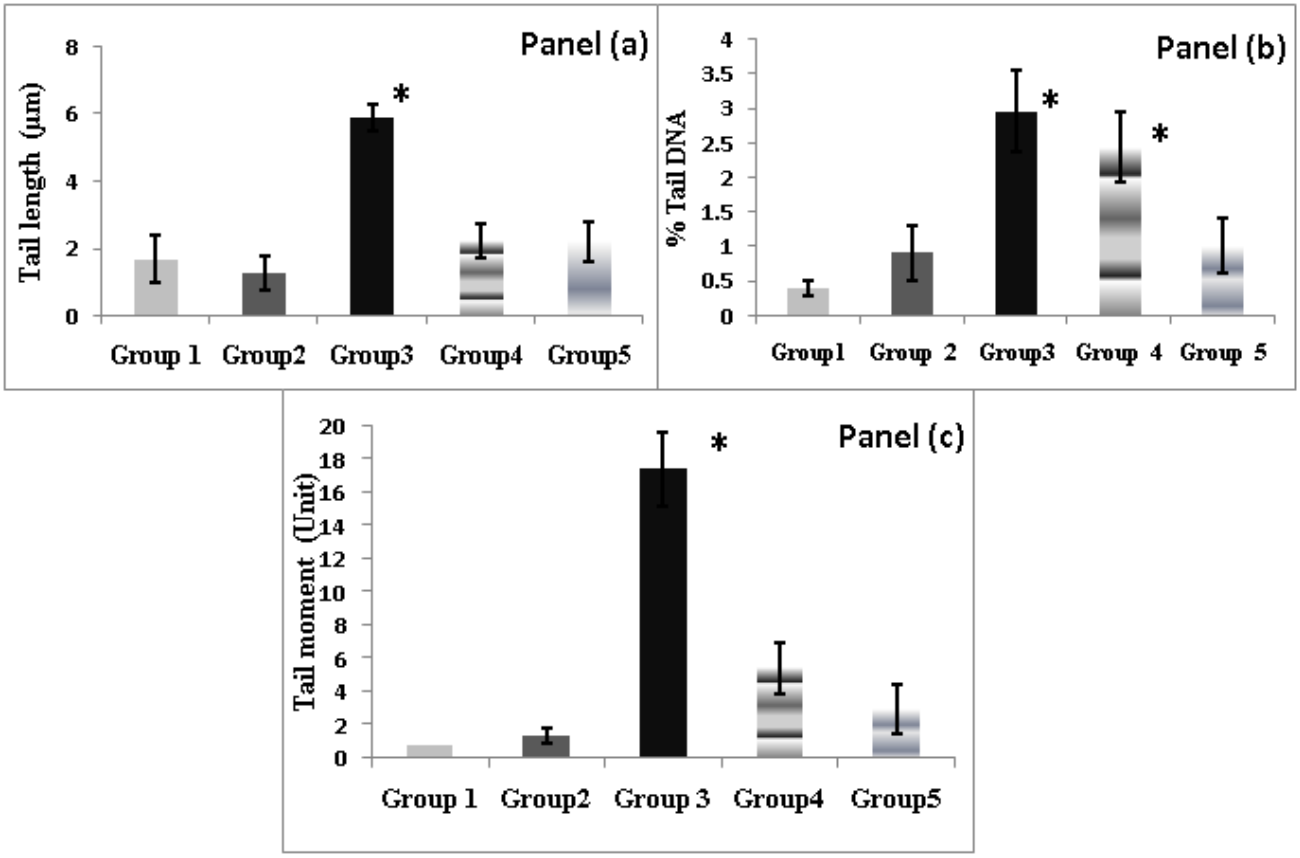

Figure 1. Comet assay parameters. Panel (a) is the tail length, panel (b) is the percentage of DNA and panel (c) is the tail moment for all the studied groups. (* Statistical significant)

Histogram of panel (a) indicated that tail length for control was 1.96± 0.7 µm then it statistically significant increased ($p<0.05$) for group 3 that treated with CCL_4 and reached a maximum length (5.88±0.4 µm). There were no statistical differences in tail length between oil group and *Spirulina*group compared to control. The mean percentage of tail DNA (panel b) reflecting the proportion of DNA that has migrated from the head, is then calculated as an average for the 50-100 cells selected for measurement. The mean percentage of tail DNA in control is 0.398±0.1 % then significant increased ($p<0.05$) was observed for groups 3 and 4.

Panel (c) illustrated the mean tail moment (whose magnitude reflects the frequency of DNA strand breaks per nucleus) for all groups compared to control. The obtained result of this parameter indicated the same phenomena for tail length that *Spirulina* reduced the degree of damage induced by CCL_4.

Image 1 and 2 of the control and oil group non-infected in which the mostly cells appeared with no comet. DNA was tightly compressed and maintained the circular disposition of the normal nucleus. Image 3 of CCl_4 group; the profile of the nuclear DNA in this group was altered with the appearance of a fluorescent streak extending from the nucleus. Image 4 of treated group with showed less damage of liver cells. Image 5 of *Spirulina* only group showed no difference with control. Degree of DNA damage observed in control group, oil group and *Spirulina*

group might be explained by the fact that about 10 000 oxidation hits to DNA per cell have been estimated to occur per day within the human body, and more than 35 different forms of oxidized bases are found in DNA in vitro [5,16]. The present result was in parallel to Kaji [22] who reported that the presence of the polysaccharide content of SP enhanced significantly both the repair activity of damaged DNA excision and the unscheduled DNA synthesis. The treatment with Sp in the current study leads to a significant reduction of DNA damage caused by CCl_4, this results were in agreement with Kaji [2] who reported that the unique polysaccharides of SP enhance cell nucleus enzyme activity and potentiate the process of DNA repair and Bhat and Madyastha [7] who found that phycocyanin and phycocyanobilin contents of Sp were also have strong anti- cyclooxygenase-2, antioxidant activity to scavenger peroxidinitrite and reduce OONO-induced oxidative damage to DNA.Most damage is repaired by effective DNA-repair enzymes, but some damage escapes repair, causing permanent damage [20].

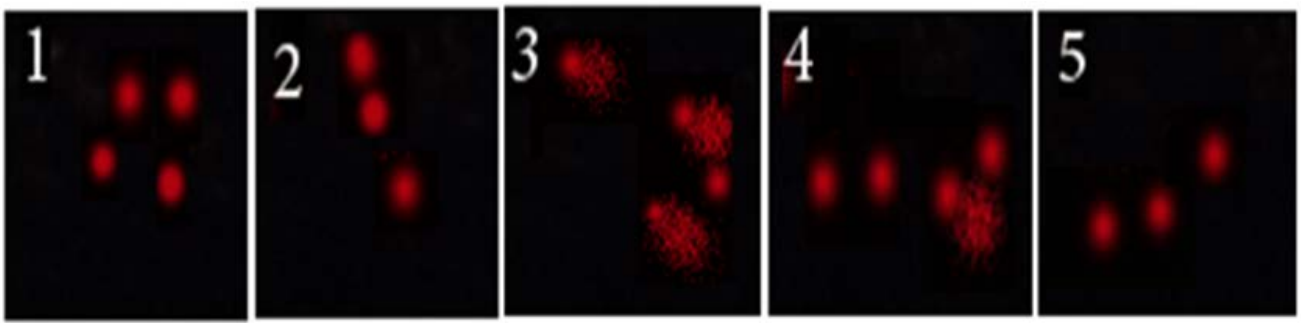

Figure 2. Comet assay images for liver cells. Image1 is the control group, 2 is oil group, 3 is CCl4 group, 4 is *Spirulina* treated group and 5 is *Spirulina* only group

4. Conclusion

In the assessment of liver damage by CCl_4, necrosis or membrane damage releases the enzyme AST and ALT from the liver into blood, and causes the level of these serum marker enzymes to increase significantly, such as that caused by viral hepatitis as well as cardiac infarction and muscle injury and hence it can be measured in the serum. AST catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. Moreover, there was an increase in circulating lipid peroxides of CCl_4 treated animals, correlates with the decline of circulatory antioxidant GPx.

Spirulina seems to preserve the structural integrity of the hepatocellular membrane as evident from the significant reduction in CCl_4-induced rise in serum enzymes and increase in MDA in mice. So, our results provide strong evidence that *Spirulina* alleviated antioxidants depletion liver, which consequently suppressed oxidative stress and improved erythrocytes and liver functions in CCl_4-intoxicated mice. However, further pharmacological evidences at molecular level are required.

Conflict of Interest

We (authors) have declared that there is no conflict of interests in the study.

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