

Original Article

Regenerative Effect of Bone Marrow-derived Mesenchymal Stem Cells in Thioacetamide-induced Liver Fibrosis of Rats

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ABSTRACT

The present study determined the regenerative effect of bone marrow-derived stem cells (BMSCs) on thioacetamide (TA)-induced liver fibrosis in rats. A total of 30 male Wistar rats were randomly divided into sham control and treatment groups. The rats of the sham control group were subdivided into three groups and sampled on the 14th, 18th, and 20th weeks after fibrosis induction. The rats of the treatment group were subdivided into two groups and sampled on the 4th and 6th weeks after BMSCs treatment. Fibrosis was induced by the intraperitoneal administration of 200 mg/kg of TA twice a week for a period of 14 weeks. All the animals underwent liver function tests and histopathologic evaluation 4 and 6 weeks after BMSCs transplantation. The BMSCs were characterized using osteogenic induction and reverse transcription-polymerase chain reaction. The BMSCs were plastic adherent, spindle-shaped, and positive for osteogenic differentiation. They expressed CD73 and were negative for CD45. The infiltration of inflammatory cells and deposition of collagen fibers were noticed after TA administration. A significant decline in inflammatory cells and a healing process were detected 4 weeks after cell transplantation. The amelioration in hepatic tissue was significant 6 weeks after cell therapy. Following the injection of BMSCs, a nonsignificant decrease was visible in aspartate transaminase level; however, this decline was significant for alanine aminotransferase level. The alkaline phosphatase and albumin levels showed an increasing trend after cell administration. The transplantation of BMSCs resulted in a significant regenerative effect after hepatic injuries. Therefore, it was shown that BMSCs transplantation can open a new window and be a therapy of choice in the amelioration of liver fibrosis.

Keywords: Bone marrow, Mesenchymal stem cell, Liver, Thioacetamide, Fibrosis, Rat

Effet Régénérateur des Cellules Souches Mésenchymateuses Dérivées de la Moelle Osseuse dans la Fibrose Hépatique Induite par le Thioacétamide

Résumé: La présente étude a permis de déterminer l'effet régénérateur des cellules souches dérivées de la moelle osseuse (BMSCs) sur la fibrose hépatique induite par le thioacétamide (TA) chez les rats. Au total, 30 rats Wistar mâles ont été répartis au hasard en groupes témoin et traitement. Les rats du groupe témoin fictif ont été subdivisés en trois groupes et échantillonnés 14, 18 et 20 semaines après l'induction de la fibrose. Les rats du groupe de traitement ont été subdivisés en deux groupes et échantillonnés aux quatrième et sixième semaines

après le traitement par BMSCs. La fibrose a été provoquée par l'administration intrapéritonéale de 200 mg / kg d'AT deux fois par semaine pendant 14 semaines. Tous les animaux ont subi des tests de la fonction hépatique et une évaluation histopathologique 4 et 6 semaines après la transplantation de BMSC. Les BMSC ont été caractérisées par induction ostéogénique et transcription inverse, réaction en chaîne avec une polymérase. Les BMSC étaient plastiques adhérents, en forme de fuseau et positives pour la différenciation ostéogénique. Ils ont exprimé CD73 et étaient négatifs pour CD45. L'infiltration de cellules inflammatoires et le dépôt de fibres de collagène ont été remarqués après l'administration de TA. Une diminution significative des cellules inflammatoires et un processus de guérison ont été détectés 4 semaines après la transplantation de cellules. L'amélioration du tissu hépatique était significative 6 semaines après la thérapie cellulaire. Après l'injection de BMSC, une diminution non significative du niveau d'aspartate transaminase a été observée; Cependant, cette diminution était significative pour le niveau d'alanine aminotransférase. Les taux de phosphatase alcaline et d'albumine ont montré une tendance à la hausse après l'administration de cellules. La transplantation de BMSC a entraîné un effet régénérateur important après une lésion hépatique. Par conséquent, il a été démontré que la transplantation de BMSC peut ouvrir une nouvelle fenêtre et constituer un traitement de choix dans l'amélioration de la fibrose hépatique.

Mots-clés: Moelle Osseuse, Cellule Souche Mésochymateuse, Foie, Thioacétamide, Fibrose, Rat

INTRODUCTION

Viral hepatitis, metabolic and autoimmune diseases, drug and alcohol abuse, and congenital abnormalities were reported as causative factors of hepatic damages (Mostaghni et al., 2011). In liver injuries, the accumulation of abundant extracellular matrix, including fibrillar collagen, occurs in the hepatocytes leading to insidious tissue fibrosis; however, most of the related morbidity and mortality happen after the development of cirrhosis (Davarpanah et al., 2009). Cirrhosis is a long-term consequence of chronic liver injury, along with fibrosis (Chen et al., 2014). Several strategies have been considered for the prevention of further damages to the liver to treat fibrosis complications and prevent liver cancer promptly (Farjam et al., 2014). Although different antifibrotic drugs, such as angiotensin inhibitors, colchicines, endothelin inhibitors, S-adenosyl-methionine, and tocopherol, were used in the treatment of liver fibrosis, there is still a limitation for these drugs as they are not efficiently taken up by activated hepatic stellate cells (HSCs) and may lead to unwanted side effects (Bataller and Brenner, 2005). Therefore, the transplantation of the liver is becoming an important option for the treatment of patients with advanced fibrosis (Dehghani

et al., 2007). Stem cells have several characteristics, such as proliferation, self-renewal, pluripotency, longevity, and differentiation, and they are a valuable source of transplantation (Ghobadi et al., 2015). It has been shown that mesenchymal stem cells (MSCs) from bone marrow (BM) could graft for repairing damaged tissues (Mehrabani et al., 2016). While the lung is exposed to bleomycin, they can ameliorate the fibrotic effects of the drug (Ortiz et al., 2007). The injection of MSCs was demonstrated to improve carbon tetrachloride (CCl₄)-induced liver fibrosis in mice (Fang et al., 2004). This may be due to MSCs effect in minimizing collagen deposition (Abdel Aziz et al., 2007). Thioacetamide (TA) is an organosulfur compound with C₂H₅NS formula that can be a potent centrilobular hepatotoxicant, undergoing a two-step bioactivation mediated by microsomal CYP2E1 to TA sulfoxide (TASO) and further to TA-S S-dioxide, a reactive metabolite that initiates cellular necrosis by covalently binding to liver macromolecules (Chilakapati et al., 2005). The experimental hepatotoxicity of TA in rodents was firstly reported in 1948, which showed that the single doses of TA within the range of 1–2 mmol/kg can lead to classical centrilobular necrosis accompanied by rises in serum transaminases and bilirubin (Kuroda et al., 1987). This study was

performed to determine the healing effect of bone marrow-derived stem cells (BMSCs) in TA-induced liver fibrosis of rat.

MATERIAL AND METHODS

Animals. Thirty male Wistar rats with the weight range of 225-250 g were obtained from the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran, and randomly divided into two groups of sham control and treatment. The sham control group (n=18) had induced liver fibrosis without treatment. The rats of the sham control group were subdivided into three groups and sampled on the 14th, 18th, and 20th weeks after the induction (Farjam et al., 2012). The rats of the treatment group (n=12) had induced liver fibrosis treated by BMSCs. The rats of the treatment group were subdivided into two groups and sampled on the 4th and 6th weeks after the treatment by BMSCs (Farjam et al., 2012). The rats were housed in an air-conditioned facility and a 12:12 h daylight/darkness cycle (light at 7:00 PM). In addition, they had access to food and water at an ambient temperature of 22±2°C and a 50% relative humidity. The study was approved by the Ethics Committee of Shiraz University (Shiraz, Iran). Blood and tissue samplings were conducted under anesthesia. Euthanasia was performed according to the ethical issues of the Ethics Committee of Shiraz University related to working with laboratory animals, and all the efforts were made to minimize suffering during the experiments.

Thioacetamide-induced liver fibrosis. Fibrosis was induced in rats as previously described by the intraperitoneal administration of 200 mg/kg of TA twice a week for a period of 14 weeks (Farjam et al., 2012). Anesthesia was performed using ketamine and xylazine.

Bone marrow-derived stem cells preparation. To isolate BMSCs, femoral and tibial bones were separated and transferred in a 15 ml falcon containing phosphate buffered saline on ice. The BM was then

flushed into a 15 ml falcon using Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) and homogenized using a pipette. The cells were plated in a culture flask containing DMEM, 10% fetal bovine serum (FBS, Bio-sera, UK), 1% penicillin and streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) (Sigma, USA), and 1% L-glutamine (Sigma, USA) and placed in a CO₂ incubator at 37 °C with 5% CO₂ and saturated humidity. The medium was replaced every 3 days, and the cells were subcultured with trypsin/ethylenediaminetetraacetic acid (EDTA, Bio-Sera, UK) at 70% confluence. To enumerate the cells at the 3rd passage, the cells were stained by trypan blue (Sigma, USA), and the number of viable and nonviable cells were counted using a hemocytometer and a light microscope. The BMSCs were morphologically evaluated by an inverted microscope (Olympus, USA).

Bone marrow-derived stem cells osteogenic differentiation. For in vitro osteogenic differentiation, the cells at 90% confluence were cultivated in DMEM, 15% FBS, 200 µM L-ascorbic acid, 10 mM glycerolphosphate, and 100 nM dexamethasone. The medium was changed twice a week for a period of 3 weeks. After 21 days, osteogenic differentiation was evaluated using Alizarin red staining. In brief, BMSCs cultures were fixed with 4% paraformaldehyde for 10 min. Then, the cells were incubated for 20 min at room temperature in 1% Alizarin Red S and 1% ammonium hydroxide. Following incubation, the cultures were washed four times, each time for 5 min with 1 ml dH₂O replacing the water at each 5-minute interval and air-dried. Alizarin Red S dye binds to calcium ions present in mineralized deposits resulting in brilliant red staining (all reagents by Sigma-Aldrich, USA).

Characterization by reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction (RT-PCR) was used in order to characterize the expression of MSC markers. Briefly, the total ribonucleic acid (RNA) was extracted by column RNA isolation kit (Denazist-Asia, Iran) as

described by the manufacturer. The total RNA concentration was assessed using spectrophotometry. From RNA samples, the complementary deoxyribonucleic acid (cDNA) was obtained using AccuPower Cycle Script RT PreMix Kit (Bioneer, Korea) based on the manufacturer's instruction. For each reaction, 15 μ L of total RNA was used to have a volume of 20 μ L with the diethylpyrocarbonate water. Twelve thermal cycles were performed, including 30 sec at 20°C for primer annealing, 4 min at 42 °C for cDNA synthesis, 30 sec at 55°C for melting secondary structure and cDNA synthesis, as well as 5 min at 95 °C for inactivation. One μ L of cDNA template was mixed with PCR buffer, dNTPs, Taq DNA polymerase, MgCl₂, H₂O, as well as forward and reverse primers (CD45: 450 bp and CD73: 208 bp; marker: 100 bp). The microtubules containing 20 μ L of this mixture were placed in the thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany), and 30 amplification cycles were performed, including 30 sec denaturation at 95 °C, 30 sec annealing at 64 °C, and 30 sec extension at 72 °C, with the 2 min at 95 °C for primary denaturation and 5 min at 72 °C for final extension. The PCR products were assessed for any defined bands using gel electrophoresis by DNA safe stain in 1.5% agarose gel medium. The bands were visualized by ultraviolet radiation and a gel documentation system (UVtec, Cambridge, UK).

Treatment with bone marrow-derived stem cells.

For cell transplantation, 1×10^6 cells/kg of BMSCs were intraperitoneally injected after the induction of liver fibrosis with TA, and the animals were sacrificed 4 and 6 weeks after the injection of BMSCs. Then, the tissue samples were transferred into formalin buffer for histological evaluation (Bruck et al., 2007).

Histological and biochemical evaluations. Tissue and blood sampling were conducted after TA-induced fibrosis, as well as 4 and 6 weeks after cell transplantation. Liver processing was performed according to previous studies using hematoxylin-eosin (H&E) staining and Masson's trichrome (Bruck et al., 2007). In brief, the liver was fixed in a 5% neutral

formalin solution, embedded in paraffin, sliced as 5 mm in thickness, and stained with H&E and Masson's trichrome. The degrees of inflammation and fibrosis were expressed as the mean of 10 different fields within each slide. For liver function tests, aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined using Randox kits (UK), and Parsazma kits (Iran) were used to evaluate alkaline phosphatase (ALP). In addition, serum albumin was determined by BioRex kit (UK).

Statistical analysis. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of serum analysis data. The differences between the mean values of the control sham and experimental groups were compared using one-way analysis of variances in SPSS statistical package for Windows (version 11.5, Chicago, IL, USA). The least significant difference was used to find significant differences considered when P-value was less than 0.05. Moreover, the comparison of histological findings between the control sham and experimental groups were compared using the Mann-Whitney test with Bonferroni correction to find significant differences at $P < 0.01$.

RESULTS

The adhered BMSCs in culture flasks displayed a fibroblast-like spindle-shaped morphology (Figure 1). Moreover, after the culture of BMSCs in differentiation medium, the cells differentiated toward osteoblasts as verified by positive staining with Alizarin red staining (Figure 2). The RT-PCR analysis of BMSCs demonstrated that these cells expressed CD73 uniformly and were negative for CD45 (Figure 3). Figure 4 depicts the histological findings of TA-induced liver fibrosis and repair in hepatic tissue after BMSCs therapy. A bridge portal fibrotic was visible after TA intoxication that resulted in the infiltration of inflammatory cells and deposition of collagen fibers in the liver parenchyma. A decline in inflammatory cells and healing effect of rearrangement of normal hepatocytes and canaliculi were detected 4 weeks after the injection of BMSCs. A significant amelioration in

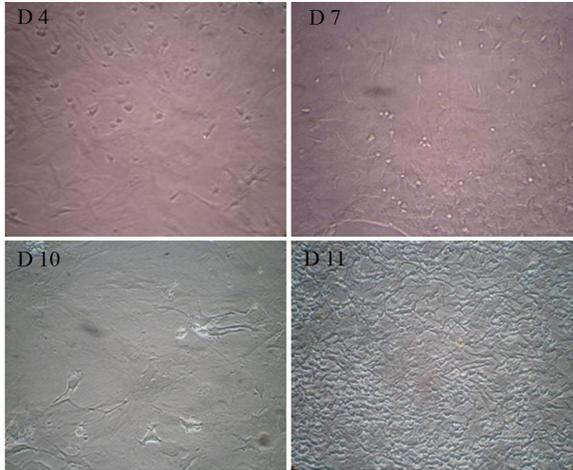


Figure 1. Bone marrow-derived mesenchymal stem cells demonstrating spindle-shaped morphology on days 4 (D4: primary culture), 7 (D7: the first passage), as well as 10 and 11 (D10 and D11: second passage)

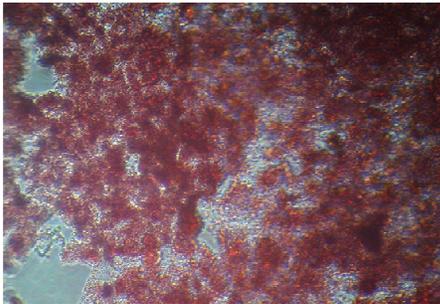


Figure 2. Cells cultivated in osteogenic medium and stained with Alizarin red at 21 days after induction (x150)

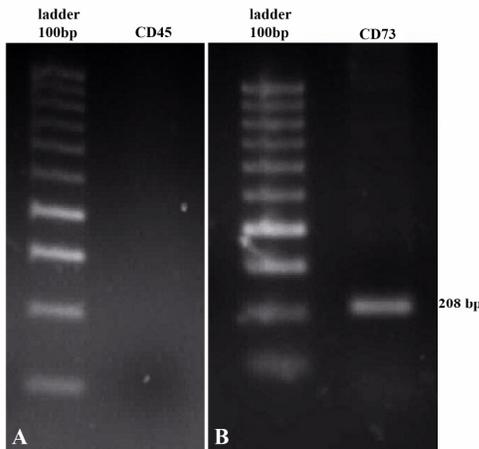


Figure 3. Bone marrow-derived mesenchymal stem cells characterized by reverse transcription-polymerase chain reaction technique; cells positive for CD73 and negative for CD45

hepatic tissue was shown by H&E staining and Masson's trichrome 6 weeks after cell transplantation (Figure 4). The scoring of fibrosis and necrosis after administration of TA (day 0), as well as 4 and 6 weeks post-transplantation of BMSCs, revealed a significant reduction in the extent of liver fibrosis ($P=0.01$). This difference was also significant between the treatment groups of 4th and 6th weeks intervals ($P=0.01$; Figure 5). The results of liver function tests demonstrated a decrease in serum AST level 4 and 6 weeks after the administration of BM-SCs when compared to TA-

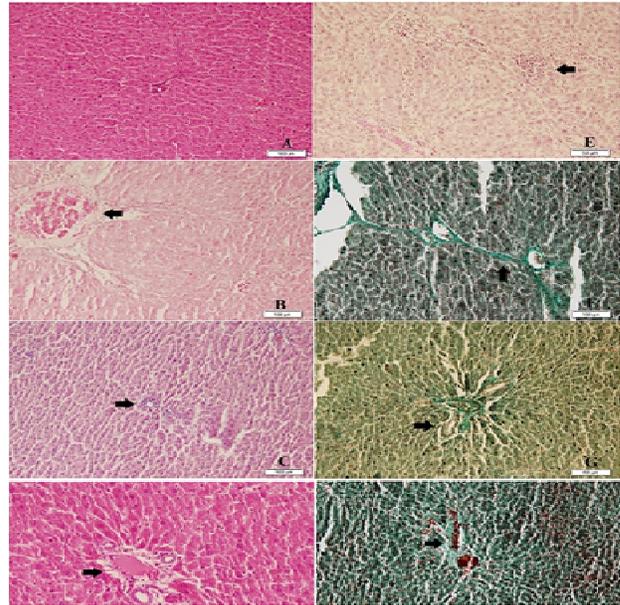


Figure 4. Histological findings after injection of thioacetamide (TA) and bone marrow-derived mesenchymal stem cells (BMSCs) in hepatic tissue; A: normal liver; B and E: interstitial and portal hepatitis after injection of thioacetamide; F and G: hepatic fibrosis due to TA intoxication; C and D: hepatic repair after 4 weeks of BMSCs therapy; H: hepatic repair after 6 weeks BMSCs therapy; A, B, C, E, and F stained by hematoxylin and eosin (x100) but F, G, and H stained by Masson's trichrome (x100); arrows indicative of changes in portal space showing a decline in inflammatory cells after cell transplantation

induced fibrosis group on the day 0, as well as after 4 and 6 weeks ($P>0.05$; Figure 6B). However, this decrease was statistically significant for ALT level 4 and 6 weeks after cell therapy ($P=0.01$; Figure 6C). The serum ALP and albumin levels showed an increasing trend 4 and 6 weeks after the injection of BMSCs;

nonetheless, the differences were not statistically significant ($P>0.05$; Figures 6A and 6D).

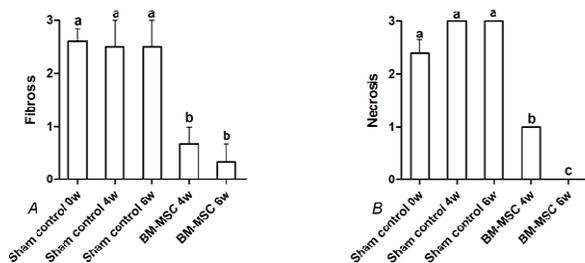


Figure 5. Histological scoring of fibrosis and necrosis in liver after administration of thioacetamide and transplantation of bone marrow-derived mesenchymal stem cells; different superscript letters showing significant differences between groups ($P<0.05$).

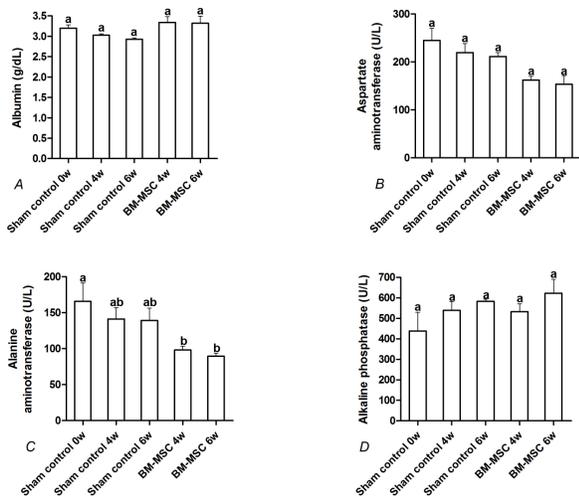


Figure 6. Comparison of liver function tests between thioacetamide liver fibrosis (i.e., sham control) and liver fibrosis induced treated with bone marrow-derived mesenchymal stem cells 0, 4, and 6 weeks after induction; different superscript letters showing significant differences between groups ($P<0.05$).

DISCUSSION

Liver fibrosis is caused due to the excessive accumulation of extracellular matrix (Okura et al., 2015). The activation of HSCs during liver injury results in the secretion of proinflammatory cytokines and increased deposition of extracellular proteins, leading to fibrosis and final destruction of liver

architecture and loss of liver function (Dekel et al., 2003). Using a rat model could successfully induce hepatic fibrosis and evaluate the efficacy of the transplantation of BMSCs in the treatment of liver fibrosis. The findings of the present study showed that BMSCs could ameliorate fibrosis in TA-induced model of rat fibrosis. The treatment of liver fibrosis with BMSCs leads to a significant reduction in the number of inflammatory cells and collagen deposition in the hepatic parenchyma. Liver function tests denoted a decrease in serum AST, and ALT levels indicated the healing effect of transplanted BMSCs; however, this decrease was mild for albumin level. In addition, an increase in ALP level was noted due to the persistent effect of TA. In fibrosis treatment, the goals are preventing activation or enhancing apoptosis of activated HSC (Piryaei et al., 2011). The secretion of a number of bioactive factors by MSCs can provide a microenvironment for the rearrangement of liver injuries (Togel et al., 2007). These factors can inhibit scarring (i.e., fibrosis) and apoptosis, promote angiogenesis, and stimulate host progenitor cells for division and differentiation into functional regenerative units (Mehrabani et al., 2013). Furthermore, the trophic effects of MSCs can have prominent clinical use (Mehrabani et al., 2016). In a mouse model of liver fibrosis, it was shown that the systemic injection of BMSCs could rescue the diseased phenotype (Okura et al., 2015). The evaluation of BMSCs effect on the liver structure in CCl_4 -induced liver fibrosis in rats demonstrated an improvement in the histological picture of the liver and its enzymatic profile (Ahmed et al., 2014). In transplanted encapsulated human MSCs in the mouse model of liver fibrosis, it was observed that MSC-derived soluble molecules were responsible for antifibrotic effects (Meier et al., 2015). The effect of BMSCs on hepatic fibrosis was evaluated in a TA-induced cirrhotic rat model, and the results showed that the treatment with BMSCs could attenuate hepatic fibrosis (Jang et al., 2014). In a mouse model of liver injury, the paracrine and endocrine effects of BMSCs were assessed, reporting the efficacy of these cells in

the amelioration of liver damages (Huang et al., 2014). The findings of this study are also in agreement with the results of the above-mentioned studies. The generation of high levels of reactive oxygen species by TA, similar to other hepatotoxins, such as CCl₄, not only resulted in tissue damages but also continued to exert effects on the transplanted cells (Kuo et al., 2008). However, it was shown that BMSCs had superior resistance to oxidative stress in vitro and in vivo (Kuo et al., 2008). There are several reports suggesting that progenitor cells from the BM can migrate to the injured liver and differentiate stellate cells and myofibroblasts. In a male to female BM transplant, it was observed that 70% of fibrogenic myofibroblasts were derived from transplanted marrow cells (Russo et al., 2006). However, there is no evidence to show any collagen expression in the injured liver caused by BM-derived cells (Higashiyama et al., 2009). The obtained results of the present study revealed that the systemic injection of BMSCs in rats suffering from hepatic fibrosis due to TA intoxication could significantly improve liver architecture and liver enzyme profiles. Therefore, based on the antifibrotic potential of BMSCs, the promising therapeutic effects of these cells may be beneficial in the treatment of patients with liver fibrosis.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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