THE EFFECTS OF MESENCHYMAL STEM CELLS AND THEIR DERIVED CONDITIONED MEDIA IN A HEPATOCELLULAR CARCINOMA MODEL

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Abstract

Background: Hepatocellular carcinoma (HCC) is the second greatest cause of cancer-related fatalities globally, with few possible treatments for studied cases with advanced HCC. As a result, there is a strong desire to develop novel alternatives therapies to cure HCC. Mesenchymal stem cells (MSCs) and MSC-derived conditioned medium (CM) has proven to be a fruitful strategy. The current research aimed to investigate role of human umblical cord MSCs (huc-MSC) & their derived CM in alleviation of HCC and clarifying related molecular mechanisms.
Methods: Rats were formed into 4 groups: normal controls (group I), HCC group (II), HCC rats which received Mesenchymal stem cells from human umbilical cord blood (huc-MSCs group III), HCC treated with huc-MSCs-CM (CM group IV). Relative expression of hepatic miR 19a and the mRNA expression of PTEN were investigated using quantitative real-time PCR.
Results: The administration of huc–MSCs and CM in HCC groups significantly improves liver function, histological appearance, and downregulate expression of hepatic microrna (miR) 19a with upregulation PTEN expression in relation to HCC group.
Conclusion: Huc-MSCs and their CM improve HCC by downregulation of miR 19a and upregulation of PTEN.

Key words: human umblical cord mesenchymal stem cells, hepatocellular carcinoma, microrna and conditioned medium.

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Background:

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the second most prevalent cause of cancer-related death worldwide (1). Despite advancements in medicinal, locoregional, and surgical therapy, HCC has high incidence, high mortality, GVNB, and frequent recurrence (2).
Mesenchymal stem cells (MSCs) have been touted as powerful therapeutic agents for a number of diseases. Human umbilical cord MSCs (huc-MSCs), one of the MSC subtypes, have received the most attention due to cause minimal harm to donors and their accessibility from a variety of sources. Significant potential exists for huc-MSCs to improve the efficacy of liver disease treatment (3).
The cell-free tumour therapy utilised UCMSCs' conditioned media. The conditioned medium (CM) of UCMSCs comprises a variety of unique exosomes, growth factors, and cytokines (4).
MiRNAs are small non-coding RNAs. They have been demonstrated to serve as tumor-promoting or tumor-suppressing genes. Differential miRNAs expression has been researched as possible indicators and targeted therapies in HCC (5).
MiR-19a downregulation led to overexpression of phosphatase and tensin homolog (PTEN), reduce cell viability, increase cell death, promote apoptosis and reduce cell invasion in HCC cells (5).
Subsequently, we searched for MSCs, their derived CM and molecular effects on the development and course of HCC through miR 19a, and related gene PTEN.

Material and techniques

Research was done in Clinical Chemistry & Stem Cell study Laboratory in Medical Biochemistry & Molecular Biology Department, Zagazig University, Faculty of Medicine, Egypt. All experimental procedures were followed guidelines of Institutional Animal Care.

Preparation of experimental animal model

Healthy male albino rats (60 in number) of similar age & weight (age, approx. 4 weeks & weight,

approx. 150 gm.). Rats were approved by Animal House of Zagazig University's Faculty of Veterinary Medicine. They were housed in temperature & light-controlled environment (12 hour light/dark cycles) with free access to food & water. Animals were classified into four groups at random:

Group I (control): 15 rats received 200 mg/Kg saline intraperitoneal (i.p.) three times with 2 weeks interval for 6 weeks then olive oil orally twice per week for another 6 weeks

Group II (HCC): 15 rats received 200 mg/kg diethylnitrosamine 2(DEN Sigma-Aldrich, ST. Louis, MO, USA) by i.p. injection three times with 2 weeks interval for 6 weeks then carbon tetrachloride 1(CCI4 Sigma-Aldrich, ST. Louis, MO, USA)1:1 with olive oil orally twice per week for another 6 weeks (6).

Group III (HCC treated with huc-MSCs): 15 rats with induced HCC received single i.p. injection of 1×10^7 human umblical cord mesenchymal stem cell per 0.5 ml PBS per rat.

Group IV (HCC treated with huc-MSCs-CM): 15 rats with induced HCC and received single i.p. 0.5 ml injecton of huc-MSCs conditioned media obtained from huc-MSC.

Histopathological examination of two sacrificed rats revealed HCC. Following that, we began intervention and treatment. Animals were sacrificed by cervical dislocation at end of research. Approximately 4-5 ml of blood was gathered from abdominal aorta of animal from rats of all groups & liver tissues were harvested for assessment of following

Gene expression of PTEN and miRNA 19a were measured by quantitative real-time polymerase chain reaction, & serum levels of AFP, ALT, AST& albumin were estimated using colorimetric methods. The liver tissues were examined histopathologically.

Isolation & culture of mesenchymal stem cells

After receiving informed consent from their husbands, cord blood was obtained under complete aseptic conditions from umbilical vein of 5 consecutive delivery women at Zagazig University Hospital. Samples were processed in accordance with procedure outlined by Kern et al., (7). Density gradient separation method was used to isolate mononuclear cell fraction, which was then cultured as per process outlined by (8). The MSCs were cultured, passaged, & propagated in accordance with (8).

Preparation of conditioned media from huc-MSC

The cell suspension was resuspended in full culture medium (Lonza Bioproducts, elevated glucose DMEM, 4.5 g/L glucose with L-glutamine,Belgium) containing ten percent FBS, one percent penicillin, and one percent streptomycin (Lonza Bioproducts, Belgium).The mixture was cultured into 25 cm2 flasks then, gently winded with uniform percussion. Cells were incubated at thirty seven°C at five percent humidified Co2 in Co2 incubator (Heraeus, Germany) then CM prepared according to (9).

Flow cytometric analysis of MSC surface biomarkers

This method was done in collaboration with clinical pathology department, Zagazig University. Fluorescence acquisition was performed using Calibur Becton Dickinson flow cytometer with CellQuest software. Determination of surface markers of UC-MSCs was done by evaluating the positive expression of CD105 and CD106 (cluster of differentiation (CD) & negative expression of CD11b surface marker (10). Pharmingen/Becton Dickinson supplied all monoclonal antibodies (Flanklin Lakes, NJ, USA).

Histological analysis

Dehydrated liver tissue samples were cleaned up in xylene & embedded in paraffin to produce tissue blocks, which were then sectioned (4-5 m) & stained with hematoxylin & eosin (11).

Real-time PCR

Real-time analysis has been reported (12). Total RNA, such as miRNAs, was separated from ready liver samples & cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, USA), Sangon Biotech (Beijing, China) for Primer assays, and TOPrealTM qPCR 2X PreMIX (Applied BiosystemsTM, USA) (SYBR Green with low ROX) (Cat. # P725 or P750) were used to examine miR-19a expression, rt-primer (5'->3') GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACAGTGCA, forward (5'->3') GTATACTCGTTTTGCATAGTT, and reverse (5'->3') GTGCAGGGTCCGAGGT (Enzynomics, Korea) in accordance with the company's protocol.

An initial denaturation at ninty five°C for twelve minutes was followed by forty cycles of decomposition at ninty five°C for twenty seconds, annealing at sixty°C for thirty seconds & addition at seventy two°C for thirty seconds. Sangon Biotech created oligonucleotide-specific primers (Beijing, China).

Regarding PCR amplification, melting curve analysis was performed. For normalisation, small nuclear RNA U6 was used (rt-primer (5'->3') AACGCTTCACGAATTTGCGT, forward (5'->3') CTCGCTTCGGCAGCACA, and reverse (5'->3') AACGCTTCACGAATTTGCGT. PTEN expression analyzed, since explained by manufacturer. For normalisation, gene for housework enzyme glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was employed. **Table 1.**

Table 1: Lists primers that were used. Rotor-Gene Q 2plex Real-Time PCR System was used for real-time PCR (Qiagen, Germany).

Gene	forward primer	reverse primer	Commission number
PTEN	ACCAGGACCAGAGGAAACCT	CCTTGTCATTATCCGCACGC	NM_022265.2
Gapdh	GCATCTTCTTGTGCAGTGCC	GGTAACCAGGCGTCCGATAC	NM_017008.4

Estimation of Alpha fetoprotein (ng/ml) serum levels

By ELISA kits (USCN Life Science Inc., Houston, TX, USA). as stated by manufacturer`s recommendations.

Estimation of serum ALT, AST, (U/L) and albumin levels (g/dl)

By routine laboratory colorimetric technique using commercial available kits Spinreact kit purchased from SPINREACT,S.A./S.A.U,SPAIN.

Statistical Analysis

Data were coded & entered into SPSS version twenty two statistical package. For numerical variables, data were summarized using mean SD. To compare groups, analysis of variance with multiple comparisons post hoc examine was used. P-values less than 0.05 were considered statistically significant.

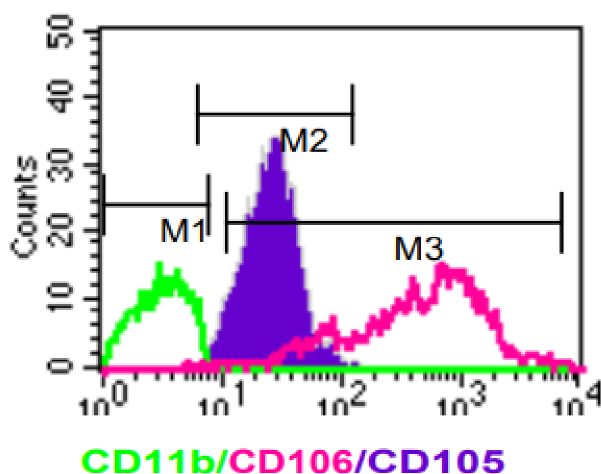
Results

Examination of stem cells in culture

Proliferation of MSCs in culture was assessed by repeated examination under inverted microscope during the culture duration in Stem Cell lab at Biochemistry department. Immediatly following inoculation, the cells were circular in shape . On the third day and till the end of the first week of culture, cells showed different sizes and shapes (oval, rounded & elongated). Cells were then allowed to proliferate; form small colonies which progressively enlarge to reach 80-90 % confluency at about 14 days of culture.

Detection of cell surface markers by flow cytometry

Flow cytometry revealed that huc-MSCs were negative for expression of hematopoietic markers like CD11b. However, they were positive for CD105, CD106 which are generally considered to be markers of MSCs. This indicated that these cells were hUC-MSCs. (**Figure 1**).



Marker	% Gated	% Total
All	100.00	39.26
M1	0.18	0.07
M2	99.97	39.25
M3	95.11	37.34

Figure (1): A: Flow cytometric analysis of cell surface markers of human umbilical cord blood mesenchymal stem cells.

Histological assessment of Liver

Control -ve group showed normal cytoarchitectures hepatic cords and cental veins, control +ve group of HCC showed pleomorphic hepatocytes, increase number of binucleated cells. Some neoplastic nuclei were hyperchromatic and other was vesicular. Large number of degenerated cells was also detected (fig 2). Group treated with huc-MSCs showed apparently normal hepatic parenchyma. Treated group with huc-MSCs-CM showed apparently normal hepatic

parenchyma, apoptotic cells and necrotic cells in some hepatic cells (fig.2).

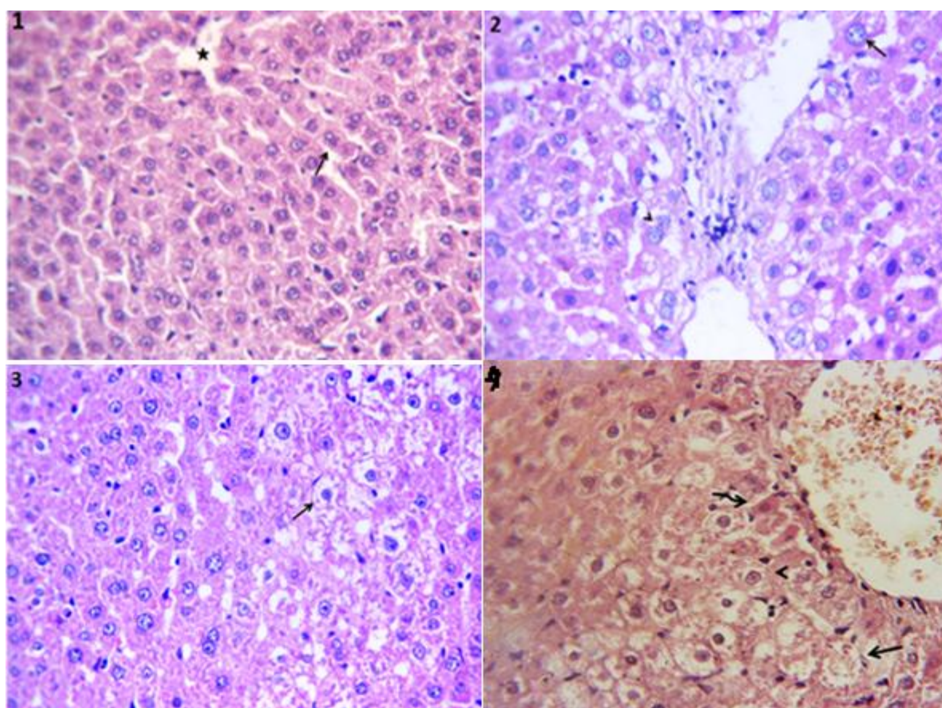


Figure (2): Photomicrograph of liver showing : 1: Normal cytoarchitectures hepatic cord (arrow) and cental veins (star) in control –ve group. **2:** Pleomorphic hepatocytes, increase number of binucleated cells (arrowhead) and hyperchromatic nuclei (arrow) in control +ve group of Hcc. H&E 40X. **3**: Focal areas of hydropic degenerated cells (arrow) parenchyma in treated group with huc-MSCs. H&E 40X. **5**: Hydropic degenerated cells (arrow head), apoptotic cells (arrowhead) and necrotic cells (arrow) in some hepatic cells in group treated with huc-MSCs-CM. H&E 40X.

Liver function test and Serum AFP

There was elevation in ALT& AST accompanied by a significant reduction of albumin in HCC group compared to control one (P<0.0001). Transplantation of huc-MSCs in HCC groups significantly decreased ALT (P<0.0001), AST (P<0.001) and significantly raised albumin in huc-MSCs & CM-group (P<0.001) in comparison with HCC group. **Table 2**.

Serum AFP was elevated in HCC group in relation to control group (P<0.01), also there is reduction in serum AFP after administration of huc-MSCs in relation to HCC group (P<0.05). In huc-MSCs-CM treated group, AFP showed significant decrease with p<0.05. **Table 2**

Table (2): Liver function test and Serum AFP among studied group:

	Control	HCC	HCC+huc-MSCs	HCC+MSC CM
ALT U/L	54.21± 3.72^{e}	137.80± 7.67^{a}	89.87± 6.43^{d}	114.50± 5.61^{b}
AST U/L	66.02± 2.45^{e}	118.11± 5.66^{a}	92.11± 4.25^{d}	109.21± 6.05^{b}
Albumen g/dl	4.20± 0.20^{a}	2.39± 0.14^{e}	3.20± 0.23^{b}	2.63± 0.07^{d}
AFP ng/ml	0.25± 0.26^{e}	1.82± 0.62^{a}	0.71± 0.40^{d}	1.10± 0.40^{b}

HCC: Hepatocellular carcinoma, **huc-MSCs:** human umblical cord mesenchymal stem cells, **CM:** conditioned medium. Different superscript letters denote statistical significance (p <0.05).

Relative expresion of hepatic miR 19a

There was upregulation in miR19a in HCC group than control group (P<0.0001). There is marked reduction in the expression of hepatic miR 19a in huc-MSCs-CM and huc-MSCs treated group (P<0.0001) in relation to HCC group. Moreover,

treated group with huc-MSCs-CM showed significant elevation of miRNA 19a than huc-MSCs treated group (**Figure 3**).

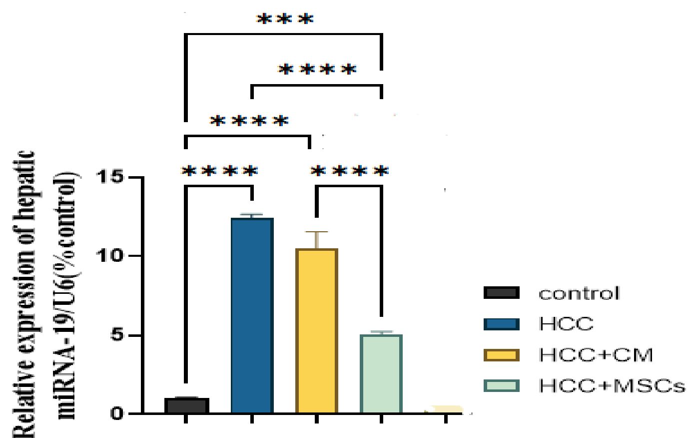


Figure (3): Hepatic expression of miR 19a

Hepatic expression of PTEN

Important downregulation of PTEN in HCC group when compared with control group (P<0.0001).There is upregulation of PTEN (P<0.001) in HCC+ huc-MSCs in relation to HCC group. Huc-MSCs-CM showed no variation when compared to HCC group (**Figure 4**).

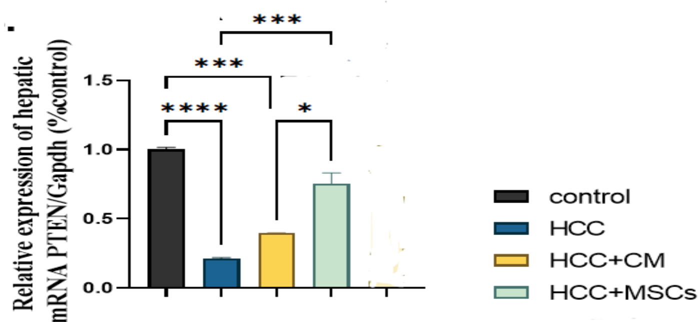


Figure (4): Relative hepatic expression of PTEN.

Discussion:

Hepatocellular carcinoma (HCC) is one of the most prevalent causes of cancer-related death worldwide (13). Unfortunately, the majority of drugs used to treat HCC today are ineffective and often even hazardous. Therefore, it is critical to find a therapeutic approach that is both secure and efficient for treating HCC (14).

In the current work, we looked at the role of huc-MSCs and their conditioned media in the therapy of HCC. MSCs therapy has been referred to be an alluring cutting-edge therapeutic approach for cancer and liver illnesses (15). Treatment with MSS-CM has been demonstrated to reduce HCC growth, promote apoptosis as well (16).

Huc-MSCs were studied because it is thought that they would make strong candidates for the clinical use of allogenic MSC-based therapies because they are simple to separate from umbilical cord blood, cultured or adapted in vitro experiments, and autologously donated into recipients, overcoming the challenges of immune rejection of transplanted cells. In comparison to bone marrow MSCs, the "gold standard," huc-MSCs shown a stronger proliferation potential and were capable of differentiating into adipogenic, osteogenic and chondrogenic tissues (17& 18).

Interestingly, we found that the animals group that administrated huc-MSCs after induction of experimental HCC showed improvement of liver function test manifested by reduced ALT, AST, and significantly elevated albumin, also, serum AFP was decreased. Moreover, histopathological picture showed apparently normal hepatic parenchyma. However, Focal areas of hydropic degenerated cells were seen. These findings show that MSCs can suppress tumour growth in chemically induced hepatocarcinogenesis.

Abdel aziz et al. discovered in their study that administering MSCs to rats after the induction of experimental HCC improved the histological image with minimum irreversible liver cell damage and improved liver function, which is similar to our findings (19).Tang et al. reported that huc-MSCs suppress the growth of HepG2 cells and promote their apoptosis and downregulate of mRNA and protein expression of genes linked to apoptotic signalling pathways as AFP, Bcl2, and Survivin (20).

The anticancer effects of huc-MSCs may also be a result of spontaneous cell fusion between tumour cells and huc-MSCs, according to Yuan et al. Each whole cell is functionally independent and the cell-cell interaction comprises complicated processes, in contrast to individual molecules such as proteins and DNA/RNA (21).

Unfortunately, evidence of the harmful impacts of huc-MSCs on cancer progression was provided by Yang et al., who demonstrated that huc-MSCs differentiate to carcinoma-associated MSCs after 2 days of culture with HepG-2 conditioned medium (22). However, due to the fact that the effects of MSCs on tumour growth are still debatable and no conclusive evidence has yet been provided, caution should be exercised when employing huc-MSC as therapeutic vehicles for HCC treatment (23).

According to Gazdic et al. the administration of CM can mitigate the tumorigenic potential, immunological reactivity, and genomic instability of stem cell transplantation (24). Moreover, the evaluation of a dose-dependent effect is made possible and information about the molecular profile of the CM (growth factors, cytokines, and chemokines) could be exploited to identify potential pathways for enhancing or inhibiting a particular carcinogenic feature (25).

Our findings demonstrated that the huc-MSCs-CM effectively inhibited HCC cell proliferation & induced HCC cell apoptosis histopathology and improve the biochemical result. These results were consistent with Yuan et al's findings that huc-MSCs-CM significantly reduced proliferation of cells and immigration in HCC cells and human lung cancer in vitro (21).Liu et al. showed that the proliferation, migration, metastasis, and angiogenesis of HCC cells are inhibited by both huc- MSCs and huc-MSC-CM and this therapeutic impact may be dynamically and quantitatively tracked in vivo by bioluminescence imaging (BLI) (26).

Huc-MSC-CM also dramatically reduced cell proliferation in cancer breast (27). However, Li et al. demonstrated that huc-MSC-CM significantly promotes breast cancer cell growth through the MAPK/ERK pathways. They explained this disparity by the amounts of the MSCs, variations in the culture and experimental procedures, the kind and site of the carcinoma, or a combination of all of these variables (28).

The involvement of several aberrantly expressed miRNAs in the development of hepatocarcinogenesis was discovered. The exact regulation of miRNAs is essential for the development, advancement, metastases, and recurrence of HCC, and several liver-specific miRNAs play a crucial role in this process (29).

Therefore, in the present study, miR-19a and its target gene PTEN expression were investigated in HCC in normal and HCC groups.MiR-19a was found to be significantly elevated & expressed in HCC liver tissue compared to controls, with large decreases in expression of tumour suppressor genes PTEN. The administration of huc-MSCs and their CM significantly downregulated miR- 19a with upregulation of PTEN.

MiR-19a excessive expression has been linked to tumour size; its stage of growth, poor prognosis as well as it is anticipated that miR-19a will likely be a candidate for use as a biomarker and a novel target for therapy for diagnostics and prognostic purposes (30). MiR-19a has been discovered for regulating PTEN expression in several types of cancer. PTEN is engaged in fundamental cell functions such as apoptosis, proliferating, and arresting the cell cycle (31).

Our findings are in line with those of Jiang et al. who demonstrated that miR-19a was considerably overexpressed in HCC and could facilitate the growth, metastasis, and therapy resistance of HCC by stimulation the PTEN/Akt signalling pathways (32). Furthermore, miR-19a expression inhibition affected the PTEN/Akt pathway, suggesting that it could be used as a noninvasive biomarker for predicting the prognosis of HBV-related HCC. For the treatment of HCC and anti-miR-19a may offer innovative treatments (5& 33).

Conclusion: While there is no one preferred treatment for HCC, huc-MSCs & their isolated CM have shown more promising spotlight for achieving more acceptable outcomes. The therapeutic effects of them are demonstrated by improvement in the

biochemical and the histological profiles. Possible mechanisms include the inhibition of proliferation & promotion of apoptosis through downregulation miR 19a and with upregulation of tumor suppressor gene PTEN.

Abbreviations:

HCC Hepatocellular carcinoma;
MSCs Mesenchymal stem cells;
MiR MircoRNAs;
Huc-MSCs Human umbilical cord blood-mesenchymal stem cells;
ALT Alanine transaminase;
AST Aspartate transaminase,
PTEN Phosphatase and tensin homolog;

Statements and Declarations:

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Zagazig University (ZA-IACUC committee). Approval number: ZU-IACUC/3/F/105/2020

Consent for Publication: I confirm that all authors accept the manuscript for submission

Availability of data and material: Available

Competing interests: None

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Conflicts of Interest: The authors declare no conflicts of interest regarding the publication of this paper.

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