Extracellular Vesicles from Human Liver Stem Cells Reduce Injury in an Ex Vivo Normothermic Hypoxic Rat Liver Perfusion Model

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Background. The gold standard for organ preservation before transplantation is static cold storage, which is unable to fully protect suboptimal livers from ischemia/reperfusion injury. An emerging alternative is normothermic machine perfusion (NMP), which permits organ reconditioning. Here, we aimed to explore the feasibility of a pharmacological intervention on isolated rat livers by using a combination of NMP and human liver stem cells-derived extracellular vesicles (HLSC-EV). **Methods.** We established an ex vivo murine model of NMP capable to maintain liver function despite an ongoing hypoxic injury induced by hemodilution. Livers were perfused for 4 hours without (control group, n = 10) or with HLSC-EV (treated group, n = 9). Bile production was quantified; perfusate samples were collected hourly to measure metabolic (pH, pO_2, pCO_2) and cytolysis parameters (AST, alanine aminotransferase, lactate dehydrogenase). At the end of perfusion, we assessed HLSC-EV engraftment by immunofluorescence, tissue injury by histology, apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, tissue hypoxia-inducible factor 1-α, and transforming growth factor-beta 1 RNA expression by quantitative reverse transcription-polymerase chain reaction. **Results.** During hypoxic NMP, livers were able to maintain homeostasis and produce bile. In the treated group, AST ($P = 0.018$) and lactate dehydrogenase ($P = 0.032$) levels were significantly lower than those of the control group at 3 hours of perfusion, and AST levels persisted lower at 4 hours ($P = 0.003$). By the end of NMP, HLSC-EV had been uptaken by hepatocytes, and EV treatment significantly reduced histological damage ($P = 0.030$), apoptosis ($P = 0.049$), and RNA overexpression of hypoxia-inducible factor 1-α ($P < 0.0001$) and transforming growth factor-beta 1 ($P = 0.014$). **Conclusions.** HLSC-EV treatment, even in a short-duration model, was feasible and effectively reduced liver injury during hypoxic NMP.

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Liver transplantation (LT) is currently the only successful therapy for end-stage liver disease, but it is limited by the discrepancy between transplant candidates and available organs. Several new strategies are under active investigation for their potential to ameliorate the transplant process and expand the donor pool.[1]

With the advent of modern preservation solutions, static cold storage (SCS) has become the standard for organ preservation. However, along with donor pool expansion, it has emerged that SCS is imperfect in preserving suboptimal organs from the so-called extended criteria donors.[2,3]

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Conflict of interest: G.C. is named as inventor in patents related to the regenerative effects of HL SC-derived extracellular vesicles. All other authors declare no conflict of interest.

F.R., N.D.S., and V.N. performed the experiments, analyzed the data, and wrote the article. E.D. and D.R. contributed in histopathological and biomolecular analyses. G.R., G.C., N.G., F.M., F.G., S.M., and D.P. participated in surgical procedures and data analysis. M.S., G.C., and R.R. gave intellectual input for study design, analyzed the data, and revised the article.

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Normothermic machine perfusion (NMP) is an innovative alternative to SCS. Through an extracorporeal perfusion circuit, it keeps the organ at physiological temperature while continuously providing oxygen and nutrients.[4,5] At variance with SCS, NMP permits real-time monitoring of biomarkers and hemodynamic perfusion parameters, potentially allowing pretransplant organ viability assessment.[6,7] Animal studies demonstrated the superiority of NMP compared with SCS,[8-12] and a phase I study established safety and feasibility of this technique in humans.[13] Furthermore, NMP gives the unique opportunity to treat the liver with pharmacological interventions during preservation.[14]

Human liver stem-like cells (HLSC) are a population of stem-like cells resident in adult liver, which may be useful in regenerative medicine because they are easily expandable and have multiple differentiating capabilities.[15] They express several mesenchymal (CD29, CD73, CD44, CD90) and embryonic markers (Nanog, Sox2, Musashi1, Oct 3/4, Pax2), but not hematopoietic ones. Moreover, HLSC express albumin, α-fetoprotein, and cytokeratin 18, supporting their partial hepatic commitment.[15] We previously showed that HLSC could restore hepatic function and improve survival in a model of fulminant liver failure in immunodeficient mice[16] and could generate hepatic-like tissue when seeded in liver acellular scaffolds.[17]

Stem cell-derived extracellular vesicles (EV) are a heterogeneous population of cell-secreted vesicles which play a pivotal role in cell-to-cell communication; they carry specific subsets of mRNA and miRNA that regulate the behavior of target cells.[18] Many in vitro and in vivo studies demonstrated the therapeutic potential of EV, showing that their beneficial effect is comparable to that of the stem cells they derive from.[19-22]

In this study, we set up a short-duration model of ex vivo isolated rat liver NMP in which oxygen delivery was kept suboptimal through low hematocrit, to investigate whether adding HLSC-derived EV (HLSC-EV) to the perfusate would result in (i) their rapid uptake by the liver, and (ii) an appreciable reduction of hypoxic tissue injury.

MATERIALS AND METHODS

Isolation, Characterization, and Culture of HLSC

HLSC were isolated from human cryopreserved hepatocytes obtained from Lonza Bioscience (Basel, Switzerland) and characterized.[15,16] HLSC were cultured in a medium containing a 3:1 proportion of α-minimum essential medium and endothelial cell basal medium-1, supplemented with L-glutamine, 2 mM; penicillin, 100 U/mL; streptomycin, 100 μg/mL; and 10% fetal calf serum (α-minimum essential medium/endothelial-cell basal medium/fetal calf serum), and maintained in a humidified 5% CO_2 incubator at 37°C. HLSC at passages 5 to 8 and 80% confluence were used in all experiments.

Isolation of DIL-Labeled HLSC-EV

HLSC were previously labeled with the DIL Stain (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate "DiI"; DiIC18(3)) (Molecular Probes Life Technology, New York, NY)[23] and cultured. Once at 80% confluence, cells were starved overnight in Roswell Park Memorial Institute/penicillin-streptomycin medium deprived of FCS at 37°C in a humidified incubator with 5% CO_2. Supernatants were collected, centrifuged at 3500 rpm for 15 minutes to remove cell debris, and submitted to ultracentrifugation at 100000*g* for 2 hours at 4°C (Beckman Coulter Optima L-90K, Fullerton, CA). EV were collected and used fresh or stored at −80°C after resuspension in RPMI plus 1% dimethyl sulfoxide. No difference in biological activity was observed between fresh and stored EV (data not shown). Quantification and size distribution of EV were performed using NanoSight LM10 (NanoSight Ltd, Minton Park, UK).[21]

Animals

Animal studies were approved by the Ethic Committee of the Italian Institute of Health (N.1164/2015-PR; Istituto Superiore di Sanità) and conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. Male Wistar rats aged 8 to 12 weeks (200-250 g weight) obtained from Charles Rivers (Italy) were maintained on standard conditions and provided with food and water ad libitum.

Isolation of Rat Livers

Animals were anesthetized through an intramuscular injection of Zolazepam (0.2 mg/kg) and Xilazyne (16 mg/kg). After intraperitoneal heparin (1250 U) administration, a midline laparotomy was performed. The bowel was retracted to expose the liver and the hepatic pedicle. The common bile duct was cannulated with a 22-G cannula, the hepatic artery was ligated, and the portal vein (PV) was cannulated with an 18-G cannula. After sternotomy, the heart was opened to exsanguinate the animal. The liver was flushed with 40 mL of cold Celsior solution (IGL, France) through PV cannula. After perfusion, the liver was removed by transecting its ligaments, PV, common bile duct, and suprahepatic and infrahepatic inferior vena cava. It was then weighed, placed into a Petri dish filled with ice-cold Celsior solution and transported to the perfusion room.

NMP

The NMP circuit was made up of a perfusion chamber, a peristaltic pump, an oxygenator (Hollow Fiber Oxygenator D150, Hugo Sachs Elektronik) and a bubble trap. A Transducer Amplifier Module (TAM-D) and a Servo Controller (SCP Type 704) (Harvard Apparatus, Hugo Sachs Elektronik) allowed constant pressure perfusion (8-10 mm Hg) and continuous monitoring of perfusate flow (1.1-1.3 mL/min per gram of liver). The perfusion chamber was linked to a warming circuit made of a thermocirculator (Lauda) with temperature set at 37°C.

The perfusion solution consisted of phenol red-free Williams E Medium, supplemented with 11.6 mM glucose, 50 U/mL penicillin, 50 μg/mL streptomycin, 5 mM L-glutamine (all from Sigma), 1 U/mL insulin (Lilly, Italy), 1 U/mL heparin (PharmaTex, Italy), named complete Williams Medium. An isovolemic hemodilution was performed by adding 20 mL of fresh rat blood to 50 mL of complete Williams Medium, thus obtaining a mean hematocrit of 9.67 ± 0.66%. This low hemoglobin content (roughly half of what is usually employed in similar perfusion settings)[11] was devised to provide suboptimal oxygen delivery and induce a limited but progressive hypoxic injury.[24] The perfusion solution was supplemented with 99% oxygen, and 2 mEq of bicarbonate was added as a pH buffer.

Livers were flushed with complete Williams Medium, connected to the NMP circuit, and perfused at 37°C through PV cannula under controlled pressure and flow conditions during 4 hours. Heparin (500 U) was added hourly during perfusion. Bile duct cannula was connected to a reservoir to quantify bile production.

Rat livers undergoing NMP were divided into 2 groups: NMP alone (NMP group, n = 10) and NMP enriched with HLSC-EV (NMP + HLSC-EV group, n = 9). In the NMP + HLSC-EV group, a single dose of 5×10^8 HLSC-EV/g of liver was added to the circuit 15 minutes after perfusion start.[22]

Perfusate Analysis

Blood gas analysis (ABL 705L Radiometer, Copenhagen) was performed hourly during perfusion on inflow and outflow perfusate samples, testing pO_2, pCO_2, and pH. In addition, outflow samples (1 mL) were collected every 60 minutes and centrifuged at 3500 rpm for 10 minutes at 4°C; the cell-free supernatants were stored at −80°C until aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were assessed by the Biochemistry Laboratory.

Immunofluorescence and Histological Analysis

To analyze HLSC-EV uptake, 2 liver lobes were included in cryostat embedding medium (Killik, Bio-Optica) and frozen at −80°C; then serial slides were cut (3-5 μm) by a cryostat and fixed in acetone. After rinsing in phosphate-buffered saline (PBS), slides were incubated with a blocking solution of 3% bovine serum albumin and 0.1% Tween-20 for 1 hour at room temperature, and then incubated overnight at 4°C with a primary antibody directed against rat cytochrome P450-4A (Invitrogen) at 1:50 dilution. After washing in PBS, the slides were incubated with the secondary antibody Alexa Fluor 488 (Invitrogen) for 1 hour at room temperature. Finally, nuclei were stained with 4′,6-diamidino-2-phenylindole and, after washing in PBS, slides were mounted with Fluoromount (Sigma). Microscopy analysis was performed using a Cell Observer SD-ApoTome laser-scanning system (Carl Zeiss).

Two other liver lobes were formalin-fixed and paraffin-embedded, then sections were obtained from most macroscopically altered areas. After hematoxylin-eosin staining, severity of histological damage was blindly scored by an experienced liver pathologist (E.D.) according to modified Suzuki criteria, by which sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4 points and the final score is the sum of the grades for each item.[25] Apoptosis was quantified by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL assay).[26] Positive and negative deoxynucleotidyl transferase dUTP nick-end labeling cells were blindly counted on 20 microscopic fields at 200× magnification, then the apoptosis index was calculated as the ratio between the number of positive cells and the number of total cells.

Quantitative Reverse Transcription Polymerase Chain Reaction

RNA was extracted from all paraffin embedded liver samples using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Invitrogen), then quantified spectrophotometrically (NanoDrop 2000; Thermo Scientific). The subsequent analyses could be performed on more than half of the samples of both NMP and NMP + HLSC-EV groups. High-capacity cDNA reverse transcription (RT) Kit (Applied Biosystems) was used to synthesize cDNA from 400 ng/μL of RNA. Then, a Real-Time Polymerase Chain Reaction (PCR) (Applied Biosystems) was performed on triplicate cDNA samples according to the chemistry of Power SYBR Green PCR Master Mix (Applied Biosystems), using the following primers of hypoxia-induced markers[27]: (a) hypoxia-inducible factor 1-α (HIF-1α), forward 5′-TGTGTGTGAATTATGTTGTAA GTGGTATT-3′, reverse 5′-GTGAACAGCTGG GTCATT TTCAT-3′; (b) transforming growth factor-beta 1 (TGF-β1), forward 5′-TTGCCCTCTACACCAACACAA-3', reverse 5′-GGCTTGCGACCCACGTAGTA-3′. The primer of the house-keeping gene actin β was forward 5′-ACCGTGAAA AGATGACCCAGAT-3′, reverse 5′-CACAGCCTGGATGGC TACGT-3′. Comparative ΔΔCt method was used to calculate the expression levels of the genes of interest normalized to the housekeeping gene expression. One liver explant from a healthy rat (sham) was used as reference sample.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Student *t* test or analysis of variance with Sidak's multicomparison test was used where appropriate (GraphPad Prism, version 6.00, USA). A *P* value less than 0.05 was considered as statistically significant.

RESULTS

After standardization, surgical procedures showed low variability between groups. Before perfusion, liver weight, tissue temperature, warm ischemia, and total ischemia time were comparable between NMP group and NMP + HLSC-EV group. During perfusion, all organs were able to self-regulate pH, pO_2, and pCO_2, maintaining stable flow and resistance (**Table S1, SDC,** http://links.lww.com/TP/B532).

Immunofluorescence analysis (Figure 1) revealed the presence of DIL-stained HLSC-EV in treated livers by the end of the experiment (Figure 1C). The colocalization of HLSC-EV with the hepatocyte marker cytochrome P450-4A demonstrated their internalization within hepatocytes (Figure 1D).

Histological analysis evidenced overt damage in the NMP livers, characterized by areas of necrosis and apoptosis, which were reduced in the NMP + HLSC-EV livers (Figures 1E and G). Tissue injury quantification by the Suzuki score (Figure 1F) showed a significant decrease in the NMP + HLSC-EV livers (3.9 ± 0.4) compared with the NMP ones (5.7 ± 0.6) ($P = 0.030$). In particular, livers treated with HLSC-EV displayed a reduced extension of necrotic areas, which never exceeded the mild degree (data not shown). Also apoptosis was significantly reduced in NMP + HLSC-EV group (apoptosis index, 0.06 ± 0.01 vs 0.14 ± 0.03; $P = 0.049$) (Figure 1H).

Biochemical perfusate analyses showed a gradual increase of AST, ALT, and LDH levels in both NMP and NMP + HLSC-EV group throughout perfusion. The AST, ALT, and LDH levels were much higher at 4 hours than at 1 hour in each group ($P < 0.0001$) (Figure 2). When compared with controls, livers treated with HLSC-EV released significantly less AST and LDH at 3 hours (AST, 92 ± 14 vs 47 ± 7 U/L per gram, $P = 0.018$; LDH: 619 ± 104 vs. 340 ± 47 U/L per gram, $P = 0.032$) (Figures 2A and B) and less AST at 4 hours (134 ± 20 U/L per gram vs 80 ± 14 U/L per gram of liver, $P = 0.003$) (Figure 2A), whereas no

differences were observed for ALT (Figure 2C). Small amounts of bile were consistently produced during the ex vivo perfusion without differences between groups (Figure 2D).

Quantitative RT-PCR analysis showed that (a) RNA expression of HIF-1α and TGF-β1 was increased in the NMP group, if compared with the sham reference sample (Figures 3A and B); (b) HLSC-EV treatment significantly reduced both HIF-1α and TGF-β1 RNA expression levels when compared with the NMP group ($P < 0.0001$ and $P = 0.014$, respectively) (Figures 3A and B).

DISCUSSION

NMP is quickly emerging as a preservation technique potentially able to improve LT outcomes using extended criteria grafts.[8-12] Thanks to physiological temperature and active hepatic metabolism, NMP also allows to pharmacologically treat the livers,[14] to reduce preservation injury or even ameliorate their quality before implantation.

HLSC are liver-resident stem-like cells, partially committed to the hepatic lineage, which carry regenerative and hepatoprotective properties.[15,16] We already demonstrated that EV derived from HLSC and mesenchymal stem cells are able to mimic most of the cell effects (including apoptosis inhibition and mitogenic activity) by transferring proteins, mRNAs, and micro-RNAs.[20,21] Possible mechanisms involved in EV effects on injured tissue include upregulation of antiapoptotic genes (Bcl-xL, Bcl2, and BIRC8) and downregulation of proapoptotic genes (Casp1, Casp8, and LTA).[18,21] Therefore, HLSC-EV may represent a treatment option for liver diseases, avoiding stem cells transplantation.

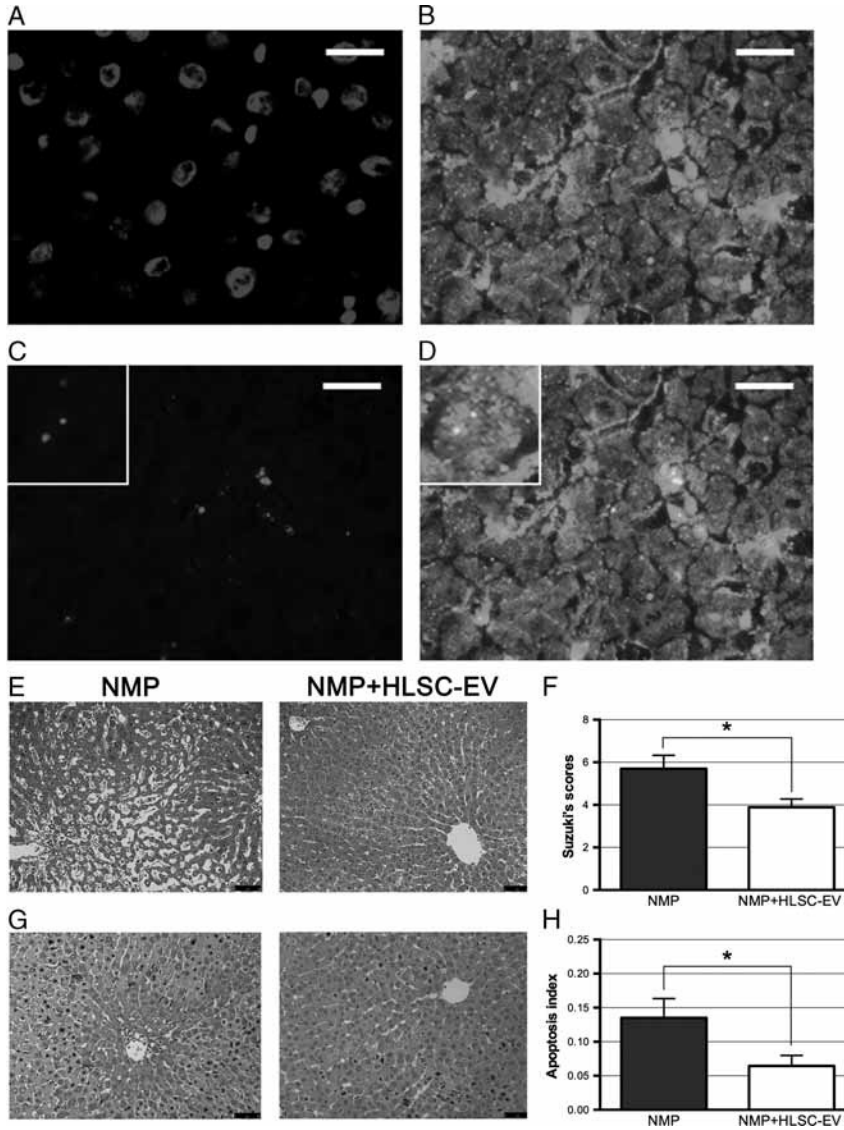

FIGURE 1. Immunofluorescence and histology in HLSC-EV–treated (NMP + HLSC-EV) (panels A, B, C, D, E, F, G, and H) and in control (NMP) (panels E, F, G, and H) rat livers after 4 hours of ex vivo isolated perfusion. Representative micrographs of fluorescence microscopy showing the cell nuclei (A), rat P450-4A immunofluorescence (B), DIL-stained HLSC-EV (C) and the merge (D) (original magnification, 630×; magnification of the insert representing the center of the image, 2520×; scale bar, 20 μm). (E) Representative micrographs of hematoxylin-eosin staining showing the grade of tissue injury (original magnification, 200×; scale bar, 50 μm). (F) Quantitative analysis for tissue damage (Suzuki score; $^*P = 0.030$). (G) Representative micrographs of TUNEL assay showing apoptotic cells (brown; original magnification, 200×; scale bar, 50 μm). (H) Quantitative analysis for apoptosis grade (apoptosis index; $^*P = 0.049$). Data are represented as mean ± SEM.

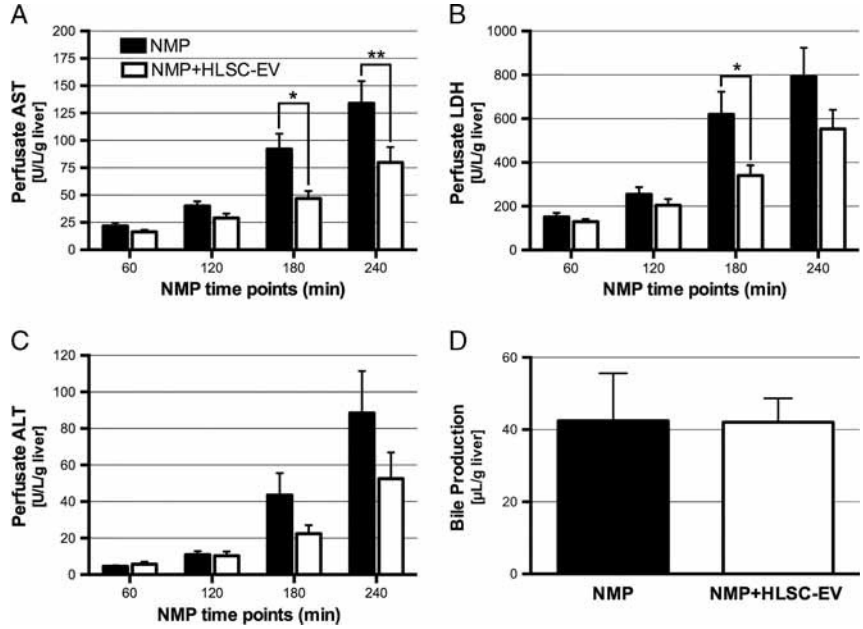

FIGURE 2. Biochemical profile of hepatic cytolysis and function markers in HLSC-EV treated (NMP + HLSC-EV) and control (NMP) rat livers assessed at different time points during ex vivo isolated perfusion. (A) AST (*P = 0.018; **P = 0.003), (B) LDH (*P = 0.032), (C) ALT, and (D) Bile production. All values are normalized to the animal liver weight in grams. Data are represented as mean ± SEM.

Combining these 2 innovative approaches, we demonstrated that HLSC-EV can be rapidly uptaken by the hepatocytes and can reduce hepatic injury in an ex vivo model of hypoxia-induced liver damage, thus providing the rationale for a pharmacological intervention with HLSC-EV during NMP.

For our purposes, we set up a simplified circuit for small organs. Because of the large body of literature on isolated rat liver perfusion,[11,28,29] an isolated-perfused rat liver model was chosen for the experimental protocol. The morphological aspect of livers after 4 hours of NMP (alone or with HLSC-EV treatment), together with the maintained bile production, evidenced that the experimental conditions allowed both survival and function of organs. Nonetheless, the preperfusion ischemic period (about 30 minutes) followed by an NMP with low hematocrit induced a limited but progressive hypoxic injury, which was proven by both the increasing levels of cytolysis enzymes in the perfusate during perfusion and the areas of necrosis and apoptosis observed in liver samples at the end of the experiments.

Fluorescent microscopy confirmed the ability of the rat liver to uptake HLSC-EV[20] and revealed the presence of HLSC-EV within hepatocytes at 4 hours. This uptake is more rapid than that observed with EV derived from mesenchymal stem cells, which were found within the injured tissue after at least 5 hours from their intravenous in vivo inoculation.[23] We hypothesize that the ex vivo isolated liver perfusion allows a faster and organ-specific distribution. To avoid modifications of hemodynamic parameters, we did not collect biopsy samples during perfusion; therefore, additional experiments are necessary to explore the precise timing of start of EV uptake within the liver during NMP.

In both groups, cytolysis enzyme levels increased with a steep slope to reach a peak at the fourth hour. This was more likely due to the insufficient oxygen delivery during perfusion rather than to an ex vivo ischemia/reperfusion phenomenon. During this injury, in the NMP + HLSC-EV group, AST and LDH levels were found to be lower at different time points, suggesting a protective effect of HLSC-EV against hypoxia.

This finding was confirmed by better histological integrity of the hepatic parenchyma and by halving of apoptosis which were observed in livers treated with HLSC-EV.

Finally, the RNA overexpression of hypoxia-induced markers was significantly reduced by HLSC-EV treatment.

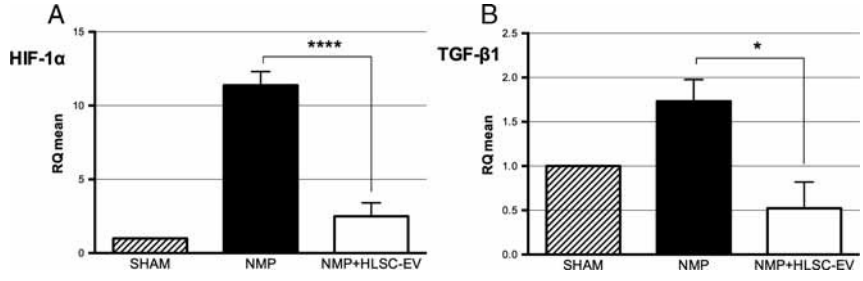

FIGURE 3. Real-time RT-PCR mean relative quantification (Relative Quantification Mean) of RNA expression of hypoxia-induced markers in a sample of rat livers after 4 hours of ex vivo isolated perfusion. Gene level expression in sham (n = 1), control (NMP, n = 6), and HLSC-EV–treated (NMP + HLSC-EV, n = 5) rat livers of (A) HIF-1α (****P < 0.0001) and (B) TGF-β1 (*P = 0.014). All values are normalized to actin β. Data are represented as mean ± SEM. RT, reverse transcription.

In a study on livers perfused at 37°C with an oxygen carrier deficiency, HIF-1α gene and protein expression levels were increased.[29] In our setting, EV treatment significantly limited the HIF-1α–dependent response to hypoxia, replicating the evidence reported in a rat model of acute kidney injury.[30] This finding is consistent with the cross-talk between HIF-1 and TGF-β1 (a multifunctional cytokine involved in many cellular pathways including inflammation, apoptosis, and fibrosis), which was recently described in hypoxic hepatocytes.[27]

As a whole, our data strongly suggest a role of HLSC-EV against hypoxic injury. Studies focusing on the characterization of HLSC-EV content are needed to clarify their mechanisms of protection.

We acknowledge that our study did not include transplantation of the livers after hypoxic NMP, yet they were overtly damaged and unsuitable for LT. We aimed indeed to investigate whether HLSC-EV could reduce hypoxic injury, a necessary proof of concept before proceeding to other experiments on organ preservation/reconditioning in normoxic conditions.

In conclusion, this study demonstrates that the NMP system can deliver stem cell–derived products to an ex vivo perfused liver and suggests that NMP + HLSC-EV could represent an innovative approach to recondition organs before transplant. Further investigations on NMP models using HLSC-EV in other experimental conditions, including transplantation, are now warranted.

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