**Original Article**

**Canine mesenchymal stem cells show antioxidant properties against thioacetamide-induced liver injury in vitro and in vivo**

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**Aim:** To overcome current limitations of therapy for liver diseases, cell-based therapies using mesenchymal stem cells (MSC) have been attempted through basic and clinical approaches. Oxidative stress is a crucial factor in hepatology, and reactive oxygen species (ROS) are well-established molecules responsible for its deleterious effects. The antioxidant properties of MSC were recently demonstrated, and therefore we examined the antioxidant activity of canine MSC (cMSC), their effects on isolated hepatocytes in vitro and their curative potential against thioacetamide (TAA)-induced liver injury in vivo.

**Methods:** To evaluate the ability of cMSC to challenge oxidative stress, cell viability, cytotoxicity and ROS were measured in cultured cMSC treated with TAA. Also, cMSC were co-cultured with hepatocytes in the same injury condition, and the ROS level was measured exclusively in hepatocytes. Finally, to verify the curative potential of cMSC, 2.0 × 10^6 cells or phosphate-buffered saline were injected systemically in non-obese diabetic/severe combined immunodeficiency mice that received TAA injections twice a week for 13 weeks. We then evaluated histological parameters, serum injury markers and redox homeostasis.

**Results:** cMSC overcame TAA-induced oxidative stress in vitro, as shown by increased viability and lower cytotoxicity and ROS levels. Moreover, hepatocytes co-cultured with cMSC also showed decreased cellular ROS. The in vivo study showed that mice treated with cMSC presented with an ameliorated histological pattern, suppressed fibrosis, lower serum injury marker levels and better oxidative parameters.

**Conclusion:** We concluded that cMSC injection reduce TAA-induced liver injury through antioxidant activities and hepatoprotective effects, showing a curative potential in liver diseases.

**Key words:** liver, mesenchymal stem cells, NF-E2-related factor 2, oxidative stress, reactive oxygen species

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**INTRODUCTION**

Liver diseases are highly prevalent in the population worldwide. Currently, despite different alternatives that have been tested, the standard treatment for end-stage chronic liver disease that is available and effective is whole liver transplantation. However, liver transplantation has serious limitations such as donor scarcity, immunological incompatibilities, high cost, and significant morbidity and mortality associated with the procedure.1–3 Additionally, considerable long-term side-effects have been reported.4–7 Given the inherent limitations of this treatment, alternative therapies are urgently needed.

In recent years, cell-based therapy, especially therapy using bone marrow cells (BMC), has emerged as an alternative to improve damaged liver function. An increasing number of studies have been published showing evidence of therapeutic effects of BMC in liver diseases,8–15 including clinical trials worldwide.16–20

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The interest in this particular cell niche comes from previous reports showing the presence of donor-derived cells in the liver of bone marrow transplant recipients.\textsuperscript{21,22} This observation, which has been proven in animal models,\textsuperscript{23,24} showed potential cross-talk between BMC and the liver under certain conditions. Among the different cell types found in bone marrow, mesenchymal stem cells (MSC) have shown promising results in tissue regeneration.\textsuperscript{8,12,14,25} These cells can be easily isolated from the patient, cultured, expanded and used as an autologous cell-based therapy.

Although promising results have been shown, important questions remain. For example, no consensus exists about the mechanisms of liver repair by BMC infusion. This topic constitutes one of the most debated issues in regenerative medicine.

Recently, oxidative stress has been shown to be an important factor in liver diseases such as liver fibrosis, cirrhosis, viral hepatitis, hepatocellular carcinoma and others.\textsuperscript{26–30} Oxidative stress is partly generated by reactive oxygen species (ROS), which are produced by different pathways such as NAD(P)H oxidases, xenobiotic metabolism, mitochondrial leakage and cytochrome P450 activity, which lead to hepatocyte damage through lipid peroxidation and alkylation of proteins, nucleic acids and lipids.\textsuperscript{31–33} Although the liver itself has an efficient antioxidant defense system, sometimes this system is not sufficient to repair the damage and/or an imbalance exists between oxidative stress elimination and production. MSC were recently reported to have an antioxidant ability that may contribute to oxidative stress resolution.\textsuperscript{34} Importantly, NF-E2-related factor 2 (Nrf2) has emerged as a crucial transcription factor that is capable of inducing a large array of enzymes involved in oxidative stress resolution.\textsuperscript{35,36} Maintenance of the cellular redox balance by Nrf2 has multiple activation pathways and has been shown to be essential in combating many inflammatory diseases.\textsuperscript{37–43} Some molecules such as all-trans retinoic acid (ATRA) and tert-butylhydroquinone (t-BHQ) have shown the ability to significantly reduce (ATRA) or induce (t-BHQ) Nrf2 functions, which modify the expression of antioxidant response element (ARE)-driven genes.\textsuperscript{44,45}

Thioacetamide (TAA) is one of the most popular chemical toxins used worldwide to generate experimental liver injury.\textsuperscript{36,47} Its toxicity results from its biotransformation by a mixed-function oxidase system (e.g. cytochrome P450 enzymes and FAD monooxygenases), which leads to the formation of reactive metabolites including ROS.\textsuperscript{48–53} ROS production resulting from TAA administration is related to the consequences of oxidative damage including lipid peroxidation.\textsuperscript{54,55}

Given the above concerns and the recent evidence for the effectiveness of cell-based therapy in liver diseases involving oxidative stress, we hypothesized that MSC could ameliorate the deleterious effects of TAA-induced oxidative stress injury in liver. In this study, we tested the ability of canine MSC (cMSC) to overcome TAA-induced oxidative stress in vitro and verified whether these cells could protect against oxidative stress damage in isolated hepatocytes. In addition, we evaluated whether cMSC could reduce the effects of TAA-induced chronic injury in vivo. An important note is that few studies have used cells derived from medium-sized animals. Results from such studies will be important for supporting new clinical trials.

**METHODS**

**Animals and ethics**

All animals used in this study were maintained and used in accordance with the Animal Care Guidelines of Yamaguchi University. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were purchased from Kyudo (Saga, Japan).

**Cells and culture conditions**

Canine bone marrow-derived MSC (Cyagen, Sunnyvale, CA, USA) were seeded onto 10-cm dishes (Iwaki, Tokyo, Japan) and cultured in OriCell Mesenchymal Stem Cell Growth Medium (Cyagen) supplemented with 10% fetal bovine serum (PBS; Life Technologies, Grand Island, NY, USA), penicillin (100 U/mL; Life Technologies) and streptomycin (100 μg/mL; Life Technologies) in a 5% CO\textsubscript{2} incubator at 37°C. After seeding, non-adherent cells were removed when the medium was replaced. The culture medium was changed every 2 days. Cells from the fourth to sixth passages were used in this study.

**Cellular characterization**

Adherent cells were dissociated with 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies) and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) containing 10% FBS. Then, they were washed once with phosphate-buffered saline (PBS; Life Technologies) and incubated in PBS containing 2% canine serum (AbD Serotec, Oxford, UK) for 20 min on ice. After incubation, cells were incubated for 20 min on ice with monoclonal antibodies against
CD11b (AbD Serotec), CD14-PE-Cy7 (BD Biosciences, San Jose, CA, USA), CD29-PE (Abcam, Cambridge, UK), CD34-PE (Abcam), CD44-PE-Cy7 (Biolegend, San Diego, CA, USA), CD45-e-fluor (bioscience, San Diego, CA, USA), CD90-APC (bioscience) or CD133-PerCP-eFluor 710 (ebioscience). Secondary detection of the CD11b antibody was performed using goat polyclonal secondary antibody to mouse IgG–H&L (DyLight 488; Abcam). Isotype-identical antibodies were used as controls. Flow cytometry analyses were performed utilizing Gallios equipment (Beckman Coulter, Danvers, MA, USA). Propidium iodide (PI; Sigma-Aldrich, St Louis, MO, USA) was used to exclude dead cells from analyses. Assessment of each sample was performed at least in triplicate. Data were analyzed using Kaluza software (Beckman Coulter). To confirm the differentiation potential, the cells were grown in osteogenic and adipogenic canine differentiation media (Cell Applications, San Diego, CA, USA) in accordance with the manufacturer’s instructions. After 2 weeks, lipid droplets were observed following oil red O staining (Sigma-Aldrich), and deposition of bone mineral was observed following alizarin red staining (AppliChem, Darmstadt, Germany).

**CM-Dil labeling**

Cultured cMSC were CM-Dil stained following the manufacturer’s instructions. In brief, cMSC were suspended in 2 μM CM-Dil (Molecular Probes, Eugene, OR, USA) and incubated for 5 min at 37 °C followed by an additional incubation at 4 °C for 15 min. Then, labeled cells were washed three times and resuspended in PBS. Cell labeling was confirmed with fluorescence microscopic observation.

**Monoculture system**

To evaluate the ability of cMSC to challenge TAA-induced oxidative stress, cMSC were seeded onto 96-or six-well plates (Corning, NY, USA) at a density of $1.0 \times 10^5$ cells/cm$^2$ in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 5% CO$_2$ incubator at 37 °C. Non-adherent cells were removed by washing with PBS. The medium was replaced with non-supplemented DMEM with or without 50 mM TAA with additional overnight incubation.

Cells in 96-well plates were washed three times, and the viability was measured indirectly using CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA). Cytotoxicity was measured with lactate dehydrogenase (LDH) quantification using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer’s instructions. Values were normalized using media exposed to the same culture conditions without cells. Finally, to quantify cellular ROS, cells in six-well plates were stained with the CellRox Reagent (Molecular Probes) following the manufacturer’s instructions. Stained live cells were quantified using Gallios equipment. Additionally, to reduce or increase the antioxidant response by Nrf2, cMSC were pretreated for 1 h with non-supplemented DMEM containing 10 μM ATRA (Sigma-Aldrich) or 10 μM t-BHQ (Sigma-Aldrich), respectively. ATRA is an inhibitor, and t-BHQ is an inducer of ARE-driven gene induction that is mediated by Nrf2. After incubation, the same volume of non-supplemented DMEM was added (final concentration, 50 mM TAA). After overnight incubation, we performed a viability test, LDH quantification and ROS measurement as described above.

**Co-culture system**

For co-culture, primary green fluorescent protein (GFP) positive murine hepatocytes were isolated from C57BL/6 Tg14 (act-EGFP) OsbY01 mice as previously described (with modifications). In brief, livers were perfused via the portal vein with pre-warmed liver perfusion medium (Life Technologies) at a flow rate of 6 mL/min for 5 min with additional perfusion with 0.05% type IV collagenase (Sigma-Aldrich) containing 20 U/mL deoxyribonuclease I (Sigma-Aldrich) at 37 °C. Whole livers were carefully harvested, the gall bladder was removed and further mechanical digestion was performed in a glass dish. The released cells were filtered through 100-μm nylon mesh (BD Falcon, San Jose, CA, USA) and washed twice with centrifugation at 50 g at 4 °C for 1 min. Finally, the pellet was resuspended in PBS and diluted in stock isotonic Percoll solution (GE Lifesciences, Uppsala, Sweden) with further centrifugation at 60 g for 10 min. The pellet was washed twice with PBS, and the viability and cell number were determined with a Trypan blue exclusion test (Molecular Probes). Then, approximately 3–5 × 10$^5$ isolated hepatocytes were seeded onto collagen I-coated six-well plates (Life Technologies) using rodent hepatocyte plating medium (Zenbio, Durham, NC, USA) in a 5% CO$_2$ incubator at 37 °C. After 4 h, the same number of CM-Dil-labeled cMSC was seeded in experimental plates (monoculture was used as control). After overnight incubation, non-adherent cells were removed by washing with PBS, and the medium was replaced with rodent hepatocyte maintenance medium (Zenbio) with or without 50 mM TAA.
After overnight incubation, hepatocytes in monoculture or direct co-culture were analyzed. For ROS quantification, six-well plates were stained with the CellRox Deep Red Reagent for 30 min. After staining, cells were harvested, centrifuged for 1 min at 50 g, and resuspended in PBS containing 1% FBS and 2 μg/mL PI for flow cytometry analysis. The cellular ROS levels of PI negative live GFP positive cells were analyzed. Figure 3(a–c) illustrates our experimental strategy.

**Experimental model of liver injury**

For liver injury induction, 6-week-old female NOD/SCID mice (n = 16) were given TAA (Sigma-Aldrich) injection (250 mg/kg i.p.) twice a week for 13 weeks. Beginning on the 10th week, 2.0 × 10⁶ cMSC diluted in 200 μL PBS (cell-treated group; n = 8) or the same volume of PBS only (non-treated group; n = 8) were slowly injected weekly via the tail vein using a metal hub needle (31/2′′/2) and a 250-μL syringe (Hamilton, Reno, NV, USA). Three days after the last cMSC/PBS injections, mice were killed.

**Biochemical analyses**

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and LDH were measured in duplicate using an automated analyzer for clinical chemistry (SPOTCHEM EZ SP-4430; Arkray, Kyoto, Japan).

**Histological staining**

Paraffin-embedded liver samples were sectioned (5 μm) and stained with hematoxylin–eosin and Sirius red according to standard protocols.

**Fibrosis level**

Histomorphometry was performed using an imaging system coupled to a fluorescence microscope (Biorevo BZ9000; Keyence, Osaka, Japan). The fibrosis index was estimated by the percent of the area that was stained with Sirius red of the total area of the histological fields, which were examined with a BZ Analyzer II (Keyence).

Hyaluronic acid (HA), procollagen N-terminal peptide (PIIIP) and AST/ALT ratio are valuable markers for fibrosis measurement. Here, serum HA and PIIIP levels were measured by using an enzyme-linked immunoassorbent assay (ELISA; Mybiosource.com, San Diego, CA, USA) in accordance with the manufacturer’s instructions.

**Antioxidant effects of cMSC**

Total antioxidant activity was measured in serum samples using an antioxidant assay kit (Cayman Chemicals, Ann Arbor, MI, USA), which is based on the ability of all antioxidant components in the sample to inhibit the oxidation of ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS+ by metmyoglobin. The amount of ABTS+ produced was monitored by reading the absorbance at 750 nm using Infinite M200 (Tecan, Männedorf, Switzerland). The capacity of antioxidants in the sample to prevent ABTS oxidation was compared with that of TROLOX, a watersoluble tocopherol analog. The result is presented as the percent of the maximum antioxidant activity.

Lipid peroxidation was assessed in liver tissues using a malondialdehyde (MDA) assay kit (Abcam), which is based on colorimetric quantification of MDA, a natural product of lipid peroxidation in the samples.

**Statistical analysis**

Data were analyzed using Student’s paired t-test or one-way ANOVA as appropriate. Values of P < 0.05 were considered statistically significant. Data are presented as the mean ± standard deviation.

**RESULTS**

**Cellular characterization**

The cells used in this study adhered to plastic and showed homogeneous distribution with a fibroblastoid shape (Fig. 1a). Flow cytometry analyses showed that cultured cMSC were positive for CD29, CD44 and CD90. On the other hand, these cells were negative for the pan-leukocyte marker CD45 and the monocyte/macrophage marker CD11b (Fig. 1b). These cells also had the potential to differentiate into adipogenic and osteogenic lineages (Fig. 1c,d), indicating a typical MSC phenotype.

cMSC have a high capacity to overcome TAA-induced oxidative stress in vitro

Surprisingly, when cultured in medium containing TAA, cMSC exhibited an elevated ability to resist this condition by showing lower levels of LDH release (cytotoxicity) into the culture medium (P < 0.001), consistent with better viability (MTS assay, P < 0.001) and, also, they showed a lower cellular ROS level (P < 0.001). Additionally, because the Nrf2 pathway is considered to be an important factor in oxidative stress protection and resolution,58 we used ATRA to abrogate and t-BHQ to induce the antioxidant effects mediated by Nrf2. In the presence of ATRA, pre-treated cMSC showed higher LDH release (P < 0.05), lower cell viability (P < 0.001)
and higher ROS levels ($P < 0.01$) compared to cMSC cultured in medium containing TAA only. On the other hand, t-BHQ reversed these ATRA effects (Fig. 2). Also, to verify whether ROS itself was responsible for viability changes, we added hydrogen peroxide ($\text{H}_2\text{O}_2$; Wako Pure Chemical Industries, Osaka, Japan) or $N$-acetyl-L-cystein (NAC; Sigma-Aldrich) to the cultures to increase ($\text{H}_2\text{O}_2$) or decrease (NAC) the ROS levels. Even though

![Figure 1](image1.png)

**Figure 1** Canine mesenchymal stem cells (cMSC) characterization. (a) cMSC adhered to plastic and showed fibroblast-like morphology (original magnification $\times10$). (b) cMSC used for infusion showed no CD45 or CD11b expression and were positive for CD90, CD29 and CD44. cMSC showed (c) adipogenic and (d) osteogenic differentiation potential. Bar indicates 100 $\mu$m. [ ], isotype control; [ ], CD antibody.

![Figure 2](image2.png)

**Figure 2** Canine mesenchymal stem cells (cMSC) treated with thioacetamide (TAA). cMSC resisted TAA-induced oxidative stress as verified by (a) lower lactate dehydrogenase (LDH) release into the culture medium (Cytotoxicity), (b) increased viability (MTS assay) and (c) lower cellular ROS levels (Cell Rox assay). An NF-E2-related factor 2 (Nrf2) inhibitor [all-trans retinoic acid [ATRA]] abrogated the values achieved by cMSC exposed to TAA. On the other hand, a Nrf2 inducer ([t-tert-butylhydroquinone [t-BHQ]]) showed the opposite results compared to ATRA. ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; [a] vs 0 mM TAA, [b] vs 50 mM TAA and [c] vs 50 mM TAA [ATRA]).
ROS levels changed as expected, no direct association was observed with viability at the time points tested (data not shown).

cMSC protect hepatocytes against oxidative stress in vitro

To evaluate whether cMSC could exert antioxidant effects on hepatocytes, we co-cultured both types of cells (CM-Dil-labeled cMSC and GFP positive murine hepatocytes; Fig. 3a,b). Cellular ROS in GFP positive hepatocytes was evaluated with flow cytometric analysis (Fig. 3c). Interestingly, hepatocyte monoculture in TAA showed lower levels of cellular ROS. The co-culture system induced further reduction in the cellular ROS level in hepatocytes treated with 50 mM TAA (Fig. 3d).

Cell-therapy ameliorates TAA-induced liver injury

Biochemical analyses were performed to verify the extent of liver injury in TAA-treated NOD/SCID mice by measuring serum ALT, AST and LDH levels. The cell-treated group showed reduced levels of serum ALT (non-treated group vs cell-treated group, 356.1 ± 48.1 vs 286.4 ± 69.3 U/L; \( P < 0.05 \)) and AST (non-treated group vs cell-treated group, 553.0 ± 174.9 vs 372.8 ± 71.1 U/L; \( P < 0.05 \)). LDH also tended to be lower in the cell-treated group, but the difference was not statistically significant (non-treated group vs cell-treated group, 880.0 ± 164.9 vs 695.6 ± 305.8 U/L; \( P = 0.06 \)) (Fig. 4).

In accordance, tissues harvested from non-treated mice showed more necrotic areas and increased inflammatory infiltration compared to the cell-treated group (Fig. 4d,e).

Figure 3 Co-culture with canine mesenchymal stem cells (cMSC) protected hepatocytes from oxidative stress. Murine green fluorescent protein (GFP) positive hepatocytes (green) were cultured alone or in co-culture with CM-Dil-labeled cMSC (red). (a) Bright field and (b) fluorescence microscopy of the co-culture system are shown. (c) Strategy to analyze GFP positive cells. (d) Cell ROX analysis of GFP positive cells revealed that hepatocytes have reduced reactive oxygen species (ROS) levels when cultured in the presence of thioacetamide (TAA). When cocultured with cMSC, they showed a further decrease in intracellular ROS (**P < 0.001; [a] vs monoculture without TAA and [b] vs monoculture with TAA). Bar indicates 100 \( \mu \)m.
Cell therapy improves redox homeostasis

Based on our in vitro results, we investigated whether cMSC transplantation could ameliorate oxidative stress in animals with chronic TAA infusions. High total antioxidant activity was sustained in sera collected from the cell-treated group, whereas the non-treated group showed a clear decrease in this ability (non-treated group vs cell-treated group, 26.9 ± 19.2% vs 61.4 ± 13.0% of maximum values; P < 0.001, Fig. 6a). In addition, lipid peroxidation was lower in liver tissues harvested from cell-treated mice, indicating lower susceptibility to oxidation in tissue (non-treated group vs cell-treated group, 23.8 ± 4.2 vs 19.6 ± 3.0 nmol/mg; P < 0.05, Fig. 6b).

DISCUSSION

Urine experimental models are commonly used to test new therapies for hepatic diseases, including cell-based therapy using bone marrow-derived cells, which have shown promising results. Among the different cell populations found in bone marrow, MSC have shown beneficial effects against liver disease. Furthermore, MSC have advantages such as multiple tissue sources, fast proliferation, possible use in autologous transplantation and in vitro manipulation. Also, MSC were recently shown to promote an antioxidant response in injured liver.

Despite good results in basic studies and clinical trials, the mechanism of action of these cells is still being discussed. Recently, many studies have linked oxidative stress and development of liver diseases such as viral hepatitis, cirrhosis, hepatocellular carcinoma and others. Here, we examined whether the antioxidant potential demonstrated by MSC has effects in reducing TAA-induced liver injury.

Thioacetamide is a drug that is widely used in animal models. Because biotransformation of TAA produces oxidative damage associated with liver injury and this drug is usually used for systemic infusion, we examined if MSC, which are also usually injected systemically, could provide resistance to the toxic effects produced by TAA. Surprisingly, rather than resistance alone, cMSC showed a high level of tolerance to TAA (Fig. 2). Additionally, when cMSC were pretreated with ATRA or t-BHQ, they showed opposite responses regarding cytotoxicity, viability and ROS accumulation (Fig. 2). Considering that ATRA inhibits and t-BHQ induces Nrf2 effects both in vitro and in vivo, these results indicate that cMSC have high antioxidant activity in vitro and suggest that the Nrf2 pathway may be involved in this process. Consistent with this hypothesis, Mohammadzadeh et al. recently showed that induced overexpression of Nrf2 by MSC was able to promote reduction of cell death in hypoxia, serum deprivation and oxidative stress conditions. In this study, MSC with transient overexpression of Nrf2 presented better cell viability and reduced apoptosis levels. Moreover, Gorbunov et al. showed that MSC treated with lipopolysaccharide, which induces inflammatory responses including release of ROS, induce a number of adaptive responses including induction and nuclear
Figure 5  Fibrosis quantification. (a–b) Morphometry analysis of Sirius red-stained liver samples showed that the cell-treated group had lower levels of fibrosis (original magnification ×4). Complementarily, serum collected from cell-treated mice presented lower mean values for (c) hyaluronic acid (HA), (d) procollagen N-terminal peptide (PIIIP) and (e) aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (*P < 0.05).

Figure 6 Antioxidant activity in cell-treated mice. (a) Total antioxidant activity was higher in the cell-treated group compared to the non-treated group (***P < 0.001). Additionally, (b) lipid peroxidation in liver tissue was lower in the cell-treated group compared to the non-treated group (*P < 0.05).
translocation of redox response elements such as nuclear factor-κB and Nrf2. They suggested that the prosurvival pathways that are activated in MSC in vitro could be a part of an adaptive response employed by stromal cells under injury conditions.65

A direct and specific effect of ROS in viability was ruled out using H2O2 and NAC in cultures. As expected, these molecules increased (H2O2) and decreased (NAC) intracellular ROS, but no direct relationship between viability and ROS levels was seen at the time points tested (data not shown). Additionally, to assess whether cMSC could potentially prevent oxidative stress in liver cells, we utilized a co-culture model with murine hepatocytes and cMSC. In this experiment, we found a lower ROS level in co-cultured murine hepatocytes treated with TAA (Fig. 3), suggesting a hepatoprotective effect of cMSC via antioxidant activity. Using a mouse primer for Nrf2 with no cross-reactivity against canine samples in silico, we verified the higher amount of mRNA in co-cultured hepatocytes (Fig. S1). However, unexpectedly, monocultured hepatocytes showed higher ROS levels when TAA was absent from the culture medium, suggesting that hepatocytes have a mechanism similar to cMSC in the presence of TAA. The underlying mechanisms are now under investigation.

Our above in vitro results motivated us to test cell therapy using cMSC in TAA-induced liver injury in NOD/SCID mice. In chronic TAA-induced injury, the animals that received cMSC infusions by tail vein showed better results for the biochemical parameters. The serum injury markers (ALT, AST and LDH) were reduced with successive cell infusions, suggesting protection of hepatocytes from necrosis and apoptosis (Fig. 4). Because ALT and AST are enzymes that reveal hepatocyte damage, these results strongly support our in vitro findings showing that cMSC have hepatoprotective effects against TAA-induced injury. We cannot rule out the possibility that infused cMSC may act systemically to aid the liver in its recovery. Consistent with our results and considering the possibility that Nrf2 may be involved in this process, Xu et al.66 demonstrated a delayed ALT decrease in sera from Nrf2-knockout mice after treatment with hepatotoxic. Because Nrf2 is crucial for induction of expression of a wide range of antioxidant genes, antioxidant activity may be essential for promoting liver regeneration.

As already discussed, oxidative stress plays an important role in liver injury, and some authors have recently demonstrated that cell-based therapy can be an effective treatment. Recently, Cho et al. have shown that MSC have an antioxidant potential to ameliorate acute liver injury induced by carbon tetrachloride.34 In a murine model of carbon tetrachloride-induced acute liver injury, they found increased Nrf2 activity and lower ROS, ALT and AST levels in animals treated with syngeneic MSC.

Okuyama et al. reported that transgenic mice with high expression of thioredoxin, a small redox-active protein with antioxidant effects, showed not only ameliorated liver injury but also decreased liver fibrosis.67,68 Consistent with this result, we showed that the possible antioxidant activity of cMSC reduced necrotic and inflammatory areas (Fig. 4d,e) and fibrosis levels by measuring of different parameters (Fig. 5). We also found higher concentration of matrix metalloproteinase 9 in liver tissues harvested from cell-treated group what can in part explain the results found in fibrosis analyses (Fig. S2).

In this present study, we confirmed that animals in the cell-treated group had better redox homeostasis by showing higher total serum antioxidant activity and lower lipid peroxidation in liver tissues (Fig. 6). The cMSC infusions seemed to sustain normal overall total antioxidant activity in these animals, which may explain the decreased lipid peroxidation (Fig. 6b), serum injury markers (Fig. 4a–c) and histological findings in vivo (Figs 4,5). At this juncture, we can clearly see that cMSC can act efficiently in combating oxidative stress in liver.

As far as we know, this study is the first to use a complete approach (in vitro + in vivo) to evaluate the role of antioxidant activity in ameliorating liver injury using cells from a medium-sized animal. These results reveal potent antioxidant activity and hepatoprotective effects of cMSC in vitro and in vivo and support more studies examining the antioxidant activity of stem cells to combat liver diseases.

In conclusion, we showed that cMSC can protect hepatocytes by reducing ROS damage induced by TAA both in vivo and in vitro. These results suggest a potential for MSC treatment in several hepatic diseases.

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**SUPPORTING INFORMATION**

**ADDITIONAL SUPPORTING INFORMATION** may be found in the online version of this article at the publisher’s website:

**Figure S1** Relative quantification of NF-E2-related factor 2 (Nrf2) mRNA in hepatocytes in co-culture showed higher values when compared to samples from monoculture under thioacetamide (TAA) condition (*P < 0.05*).

**Figure S2** Enzyme-linked immunoassay revealed that liver tissues harvested from cell-treated group presented higher concentration of matrix metalloproteinase 9 (*P < 0.05*).