The Effects of Placental Mesenchymal Stem Cells Labeled With Ultrasmall Superparamagnetic Iron Oxides on the Growth of Colorectal Cancer Cells

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Objective: Colorectal cancer (CRC) is one of the most common malignant tumors worldwide, with effective intervention and treatment being essential for CRC management. This study investigated the effects of human placental mesenchymal stem cells (PMSCs) labeled with ultrasmall superparamagnetic iron oxides (USPIOs) on the growth of CRC cells and the feasibility of 3.0-T magnetic resonance (MR) imaging as an in vivo tracer.

Methods: Twenty subcutaneous CRC HT-29 xenograft model in immunodeficient mice was established. Mice injected with labeled PMSCs were considered as the experimental group. Thereafter, the growth and MR signal changes of xenograft tumors of every nude mouse were measured. Then, growth curve was plotted, and the MR image quality in different sequences was analyzed. Pathological staining was performed after MR scan.

Results: Ultrasmall superparamagnetic iron oxides–labeled PMSCs had no significant influence on biological characteristics (P > 0.05). The growth of tumors in mice in the experimental group before the injection of PMSCs was similar to that of the control group. Contrarily, the tumor growth rate in the experimental group on day 5 post-PMSCs injection was slightly lower than that of the control group. Moreover, the tumor volume on day 14 was noticeably smaller than in the control group. The tracking ability of T2* mapping sequences for USPIOs-labeled cells was significantly more effective than T2-weighted image and T2 mapping sequences.

Conclusions: Ultrasmall superparamagnetic iron oxides–labeled PMSCs injected into CRC transplanted tumors can be studied for a long period of time. Furthermore, 3.0-T MRI in vivo molecular imaging was demonstrated to be effective for CRC intervention.
MATERIALS AND METHODS

Cultivation of PMSCs

Human PMSCs were donated by the Stem Cell Research Institute of the General Hospital of Ningxia University. Placental mesenchymal stem cells were cultured, and the concentration was adjusted to $3 \times 10^5$ cells/mL. Then, 100 μL cell suspension was added to Eppendorf tube, and 2 μL primary antibodies (CD29, CD105, CD73, CD11b, CD14, CD45, and IgG1) (Cyagen, Guangzhou, China) was added. Next, the culture was incubated at 4°C for 30 minutes, centrifuged, and washed, and the supernatant was removed. Thereafter, 2 μL of fluorescein isothiocyanate-conjugated secondary antibody (Cyagen) was added and then incubated in an ice box in the dark for 30 minutes, which was then centrifuged and washed with phosphate-buffered saline, and finally, the positive expression rate of cell surface markers was detected.

USPIOs-Labeled PMSCs

Prepared cells were seeded into a 6-well plate with a density of $2 \times 10^5$ cells/mL. After the cells adhered to the wall, 1.5 mL of culture medium was added and then incubated. Next, 15 μL of USPIOs and 0.45 μL poly-l-lysine (PLL) were mixed at a ratio of 1:0.03, placed in a vortex mixer at 2200 r/min, and shaken for 30 minutes to form PLL-USPIO complex. This was added to the culture medium at a final concentration of 10 μL/mL. After 24-hour incubation, the medium was fixed with 4% paraformaldehyde and stained with Perls Prussian blue (Solarbio, Beijing, China). The number of Prussian blue–stained and unstained cells were counted using a hemocytometer ($\times 100$) under an inverted microscope. Labeling efficiency was determined by percentage of Prussian blue–stained positive cells compared with the total cell number.

Comparing Biological Characteristics of PMSCs

The Trypan Blue dye (Solarbio) exclusion test was carried out to determine the number of viable cells present in a cell suspension. An equal amount of PMSCs suspension and 0.4% Trypan blue solution were mixed, and then the stained cells were counted. Because of incomplete cell membrane, dead cells stained blue, whereas the living cells remained unstained. Cell Counting Kit-8 (Dojindo, Japan) was used to assess cell proliferation. For this purpose, $2 \times 10^5$ PMSCs were seeded into 96-well plates. The labeled cells were considered as the experimental group and the unlabeled cells as the control group. The medium without cells was considered as the blank group (there were 5 duplicate wells in each group). On days 1, 3, 5, and 7 after labeling, 10 μL Cell Counting Kit-8 solution was added to the 100 μL complete medium. This was then incubated for 2 hours to detect optical density (OD) value of each well at a wavelength of 450 nm. The OD value of different groups was compared, and a plot was formed to determine the cell proliferation curve. In addition, Annexin V–fluorescein isothiocyanate/PI cell apoptosis detection kit (Kayki, Jiangsu, China) was used to detect the rate of apoptosis. This was achieved by comparing the cell densities of labeled and unlabeled PMSCs, and the cell density was adjusted to $1 \times 10^5$ cells/mL. Furthermore, 500 μL of 1× Annexin V Binding Buffer (Kayki) was added to resuspend the cells. Next, the cells were gently vortexed and incubated at room temperature for 15 minutes in the dark.

Establishment of Animal Models

Twenty healthy female BALB/c nude mice (body weight, 18–22 g; age, 4–6 weeks) were tested at the Animal Center of the General Hospital of Ningxia University. Moreover, 20 nude mice were randomly divided into 2 groups (n = 10 mice for each group). The concentration of HT-29 cells was adjusted to $10^7$ cells/mL and subcutaneously inoculated into the right groin of the nude mice. The experiment was performed on day 14 when the tumor diameter was approximately 5 mm. In the experimental group, $5 \times 10^6$ cells/mL of USPIOs-labeled PMSCs was injected into tumorous mice, whereas no injection was administered in the control group. The growth of xenograft tumors of nude mice was measured every 3 to 4 days. The growth curve was plotted to calculate the volume of tumor.

MRI Scan In Vivo

Four percent isoflurane was used to induce anesthesia for the mice, and the concentration was adjusted to 1.5% isoflurane for deep anesthesia. Scanning parameters included for different scans were as follows: for T2-weighted image (T2WI) fast spin echo sequence—repetition time (TR), 2000 milliseconds; time to echo (TE), 140 milliseconds; echo chain length, 15; flip angle, 150°; filed of view (FOV), 35 mm × 35 mm; and acquisition matrix, 176 × 176; for T2 mapping sequence—TR, 2000 milliseconds; TE n* 13 milliseconds; 6 echoes of 13, 26, 39, 52, 65, and 78 milliseconds; flip angle, 90°; FOV, 50 mm × 50 mm; and acquisition matrix, 124 × 124; and for T2* mapping sequence—TR, 28 milliseconds; TE n* 3 milliseconds; 6 echoes of 3, 8, 12, 16, 20, and 24 milliseconds; flip angle, 20°; FOV, 30 mm × 30 mm; and matrix, 100 × 96. For transverse scanning, layer thickness was 2 mm, layer spacing was 0.2 mm, and specimen were scanned before the PMSCs injection on the days 1 to 3, 7, 10, and 14, and the signal change rates were analyzed ($\Delta$SI, $\Delta$T2, $\Delta$T2*). A graph was plotted to determine the time signal value curve, the signal-to-noise ratio (SNR) was compared, and the contrast signal-to-noise ratio (CNR) and contrast (C) values of each sequence were established. The following equations were used to calculate the factors: $\Delta$SI = [(SI labeled − SI tumor)/SI tumor] × 100%; $\Delta$T2 = [(T2 labeled − T2 tumor)/T2 tumor] × 100%; $\Delta$T2* = [(T2* labeled − T2* tumor)/T2* tumor] × 100%; SNR = SI tumor/SD noise; CNR = SI tumor − SI labeled/SD noise, and C = SI tumor/SI labeled. In this study, SI labeled and SI tumor were regarded as the strength of the signal of labeled and tumor cells. SD noise is the standard deviation of the background signal strength. Bleeding and necrosis were avoided when measuring the tumor tissue. The largest section of the contralateral thigh was used to measure muscle size. The region of interest was determined by size of tumor, image background was 3 cm², and the labeled area was 0.5 cm². Three regions of interest were selected and averaged.

Pathological Assessment

Two mice were selected, one each from the experimental and control group to be sacrificed on day 2 after PMSCs injection for Perls Prussian blue staining. The remaining mice were sacrificed after MRI on day 14 after PMSCs injection. The inhibition rate of tumor growth was calculated using the following formula:

\[
\text{Inhibition rate(%) = } \frac{\text{Mean tumor weight of control group} - \text{Mean tumor weight of experimental group}}{\text{Mean tumor weight of control group}} \times 100\%
\]

Moreover, hematoxylin and eosin (H&E), Perls Prussian blue, CD31, CD34, and Ki-67 staining methods were performed. Microvascular density (MVD) was calculated by modifying the Weidner method.

Statistical Analysis

The data were statistically analyzed by SPSS 23.0 software (IBM, Armonk, NY), and all experimental data were expressed as mean ± SD. The rate of survival, apoptosis, proliferation, and tumor inhibition of labeled and unlabeled cells were compared by an independent t test. The volume of transplanted tumors was
analyzed by repeated-measures analysis of variance. The values of SNR and CNR for each sequence were compared by analysis of variance. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Identification of PMSCs**

After inoculation of PMSCs for 24 hours, the cells were observed under microscope to be flat and irregular. Results of flow cytometry analysis showed that the expression rates of CD29, CD73, and CD105 were 99.4%, 98.5%, and 96.0%, whereas CD11b, CD14, and CD45 were hardly expressed. In comparison, CD11b, CD14, and CD45 were hardly expressed (Fig. 1). The Prussian blue–stained particles were found in the cytoplasm of magnetized labeled cells observed under a fluorescence microscope. The labeling rate of the cells was >95% (Fig. 2).

**Cell Biological Characteristics**

The survival rates of the cells after 24 hours in the unlabeled group and the labeled group were 95.50% ± 0.50% and 94.69% ± 0.11%, respectively. The survival rate was compared between the 2 groups (\( t = -0.81, P = 0.58 \)), which indicated that the USPIOs-labeled PMSCs did not significantly affect cell viability. Regarding cell proliferation ability, the trend of the cells labeled with USPIOs from days 1 to 7 was similar to that of unlabeled cells, and the OD value increased over time. The \( P \) values from days 1, 3, and 5 were 0.83, 0.06, and 0.22, respectively, indicating that

![Figure 1](https://example.com/fig1.png)

**FIGURE 1.** Color graph showing identification of surface antigens in PMSCs. The expression rates of CD29, CD73, and CD105 were 99.4%, 98.5%, and 96.0%, whereas CD11b, CD14, and CD45 were hardly expressed. Figure 1 can be viewed online in color at www.jcat.org.

![Figure 2](https://example.com/fig2.png)

**FIGURE 2.** Color graph showing Prussian blue staining of magnetically labeled PMSCs (×100). The Prussian blue–stained particles were found in the cytoplasm of magnetized labeled cells, and the labeling rate was >95%. A, The labeled cell image under the inverted microscope. B, The labeled cell image under the fluorescence microscope. Figure 2 can be viewed online in color at www.jcat.org.
USPIOs-labeled PMSCs had no significant influence on the cell proliferation within a certain period of time (Fig. 3). It was revealed that apoptosis rate was not significantly different between the labeled and unlabeled group ($t = 15.40, P = 0.21$), indicating that the USPIOs-labeled PMSCs also had no remarkable influence on the apoptosis rate (Fig. 4).

**Growth of Transplanted Tumors**

The incubation period of HT-29 cells that were transplanted via subcutaneous injection was about 5 days. Cyclical mounds with a diameter of 3 mm were noted in each groin of tumor-bearing mice, and there were subcutaneous nodules with a diameter of 4 to 5 mm after 2 weeks. After 3 weeks, the nodule with a clear boundary increased to a diameter of 6 to 7 mm, and the tumor diameter was about 8 to 10 mm after 4 weeks. As shown in Figure 5, the trend of tumor growth in the experimental group before injection of PMSCs was similar to that of the control group, whereas the tumor growth rate in the experimental group on day 5 after injection of PMSCs was slightly lower than that in the control group, and the volume of tumor on day 14 was noticeably smaller than that in the control group. However, the volume of tumor was notably different between the 2 groups ($P > 0.05$).

**Changes in MR Signal**

The T2WI, T2 mapping, and T2* mapping sequences of the transplanted tumor showed high signals. On day 1 in the experimental group, T2WI, T2 mapping, and T2* mapping sequences showed bar-shaped low-signal regions. T2 and T2* values were significantly lower, whereas T2* value markedly changed in tumor tissues. The signal value was the lowest on day 2 after injection of PMSCs, and on day 3, the signal value of the USPIOs-labeled PMSCs gradually increased and the signal value in the tumor tissue was the lowest. On day 7, the region with low-signal was unclear in some tumors, and the signal values of tumor tissues and USPIOs-labeled PMSCs did not noticeably change. On day 10, the signal value of the tumor significantly increased, and changes...
of the signal value gradually decreased over time. The T2 and T2* value of tumor and labeled cells, respectively, were compared. The signal values at days 1, 3, 5, 7, and 10 were all statistically significant (P < 0.05). Changes in intensity had no obvious influence on the experiment, in which the transplanted tumor in the control group showed a high signal and the volume of the transplanted tumor increased over time. On day 14, a large area of high signal necrosis appeared in both groups (Figs. 6–8).

FIGURE 6. Gray scale image indicating different sequence imaging of transplanted mice. Lines A, B, and C were T2WI, T2 mapping, and T2* mapping images of experimental group, and ① to ⑤ were MR images of experimental group for days 1, 3, 7, 10, and 14, respectively; white arrows pointing to the bar-shaped low-signal regions were the magnetically labeled cells. (T2WI fast spin echo sequence: TR, 2000 milliseconds; TE, 140 milliseconds; flip angle, 150°; FOV, 35 mm × 35 mm; and acquisition matrix, 176 × 176; T2 mapping sequence: TR, 2000 milliseconds; TE n* 13 milliseconds, 6 echoes of 13, 26, 39, 52, 65, and 78 milliseconds; flip angle, 90°; FOV, 50 mm × 50 mm; and acquisition matrix, 124 × 124; and T2* mapping sequence: TR, 28 milliseconds; TE n* 3 milliseconds, 6 echoes of 3, 8, 12, 16, 20, and 24 milliseconds; flip angle, 20°; FOV, 30 mm × 30 mm; matrix, 100 × 96)

FIGURE 7. The time-signal value curve of experimental group and control group from 1 day, 2 days, 3 days, 7 days to 10 days; experimental group contains tumor signal value and the signal value of labeled region in tumor. It showed time-T2 value curve and time-T2* value curve, respectively.
Quality of MR Images

In the 3.0-T MR, T2 mapping and T2* mapping sequences showed different TE image quality value, in which TE value increased with significantly reduced SNR and the increase of CNR (Fig. 9). By comparing the SNR value for each sequence, the following consequences were revealed: $P \leq 0.001$ for T2WI versus T2 mapping, $P = 0.037$ for T2WI versus T2* mapping, and $P = 0.045$ for T2 mapping versus T2* mapping. The SNR of T2* mapping sequence for USPIOs-labeled PMSCs was higher than that in T2WI and T2 mapping sequence. Comparison of CNR value for each sequence unveiled the following outcomes: $P = 1.000$ for T2WI versus T2 mapping, $P = 0.015$ for T2WI versus T2* mapping, and $P \leq 0.001$ for T2 mapping versus T2* mapping. The contrast of T2* mapping sequences of USPIOs-labeled PMSCs was greater than that of T2WI and T2 mapping sequences (Table 1).

Pathological Findings

Hematoxylin and eosin staining showed that the tumor was poorly differentiated adenocarcinoma with large cancer cells, unclear cytoplasmic boundaries, thickened tumor stroma, thin-walled blood vessels, and diverse nucleus morphology. It was disclosed that, on the following day after injection of PMSCs, the Prussian blue–stained particles could be observed on the edge of tumor tissue. Besides, positively expressed CD31 and CD34 cells were found in both the experimental and the control groups, and the neovascularization was rich and distributed in the form of brown strips. The MVD in the experimental group and control group was $65 \pm 15.7$ and $62 \pm 14.3$, respectively. Comparison of positively expressed CD31 cells between the experimental group and the control group showed $t = 0.245$ ($P = 0.819$), whereas that of positively expressed CD34 cells showed $t = 0.270$ ($P = 0.800$). The MVD was consistent for the 2 microvascular endothelial markers ($P > 0.05$). The positive rate of Ki-67 was greater than 50%, and there was no meaningful influence on tumor proliferation between the 2 groups (Fig. 10).

DISCUSSION

Colorectal cancer is a serious threat to human health and has become an urgent problem for researchers in recent years. Many reports suggest that MSCs have inhibitory effects on the growth of various tumors, but its effect on colorectal tumors is still inconclusive. Therefore, this study investigated whether molecular imaging method used to magnetically label MSCs can inhibit the growth of colorectal tumors, observed by MR.

![Time-Signal change rate curve](image1)

$$\Delta SI = \frac{(S_{tumor} - S_{labeled})}{S_{tumor}} \times 100\%,$$

$$\Delta T2 = \frac{(T2_{tumor} - T2_{labeled})}{T2_{tumor}} \times 100\%,$$

$$\Delta T2^* = \frac{(T2^*_{tumor} - T2^*_{labeled})}{T2^*_tumor} \times 100\%.$$
Direct injection of USPIO into the body has poor targeting performance. A high label concentration will increase the generation of free radicals in cells, and the nanoparticles are easily oxidized in the air. Researchers generally believed that the modification of USPIO with a lower label concentration increased the activity of labeled cells, stabilized nanoparticles, and improved iron labeling efficiency. In this study, PLL, a widely used modifier, was used to transfect USPIOs. In the preliminary experiments, the concentration of PLL was too low to modify USPIO, thereby affecting the cell state. We used the ratio of USPIO/PLL as 1:0.03 and the concentration of USPIO to be 10 μg/mL to label PMSCs; the biological characteristics such as cell morphology, survival rate, proliferation ability, and apoptosis rate did not change significantly.

T2 mapping and T2* mapping used echo-signal curve fitting to form pseudocolor maps to quantify iron particles in tissues, which can indirectly reflect the iron concentration in the tissues. The signal intensity was generally affected by several factors, such as magnetic field, pulse uniformity, TR, and TE, whereas the relaxation time was relatively constant. Therefore, T2WI, T2 mapping, and T2* mapping sequences are widely used worldwide. In this study, regions with low signal intensity were noted in T2WI, T2 mapping, and T2* mapping sequences on transplanted tumors on the first day after the injection of PMSCs. The T2 and T2* values of these regions were notably lower than those in tumor tissues, indicating that the magnetically labeled PMSCs have entered the tumor tissue. With the decrease of T2* value, the signal value of the labeled cells reached the lowest on the day 2, whereas the signal value of the labeled cells gradually increased on the day 3. On day 7, the regions with low signal value narrowed, and these areas in some tumors showed poor clarity. On the 10th day, the signal value of the tumor tissue increased. On day 14, there was no significant difference between the experimental group and the control group, and a large area of necrosis was observed in the tumor. This revealed that the intensity of MR signal did not significantly change over time and could not be used as a reference, whereas the changes of T2 and T2* values were consistent with MR images, which was consistent with previous studies.

For T2 mapping and T2* mapping sequences in the experimental group, the TE and CNR value increased and the SNR decreased. This indicated that the longer TE of the magnetically labeled cells is associated with worse SNR of the image, whereas the CNR of the image improves. Therefore, selection of an appropriate TE is particularly important for medical imaging. Previous research demonstrated that the T2* mapping sequence was not significantly different from T2 mapping in the imaging and quantification of iron in the tissue. However, in our current study, it was unveiled that the T2* value was lower than the T2 value of the labeled cells in the experimental group. It showed that the T2* mapping sequence was more sensitive to the inhomogeneity of the magnetic field and was more sensitive to reflect the change of the magnetic sensitivity in the tissue. Comparing the image quality of T2WI, T2 mapping, and T2* mapping sequences on USPIOs-labeled PMSCs, we found that the SNR of T2* mapping image did not significantly differ from T2 mapping and that both were higher than T2WI sequence. T2* mapping sequences of labeled cells and tumor tissues were more evident than T2WI and T2 mapping sequences, which indicated that the T2* mapping sequences were more effective than T2WI and T2 mapping sequences in USPIOs-labeled PMSCs.

Past studies have reported that MSCs can recruit lymphocytes for cancerous tissues, inhibit cell proliferation, and promote

![FIGURE 10. Color graph showing results of pathological staining. Lines A and B were experimental group and control group, respectively. The Prussian blue–stained particles could be observed in the edge area of tumor tissue. Besides, positively expressed CD31 and CD34 cells were found in both the experimental group and the control group, and there was no significant difference in the positive expression rates of CD31, CD34, and Ki-67 between the 2 groups (P > 0.05). Figure 10 can be viewed online in color at www.jcat.org.](image-url)
cell apoptosis. However, in the present experiment, H&E staining of USPIOs-labeled PMSCs revealed that the MVD of cancer cells did not significantly increase or decrease, the necrotic area around the cancer tissue gradually increased over time, and the degree of malignancy was extremely high. Regions with low signal intensity were found in the MR images in the experimental group. Perls Prussian blue staining after scanning on day 1 confirmed the presence of USPIOs-labeled PMSCs. On day 14, no obvious Prussian blue–stained particles were found in the tumors in the experimental group and the control group, indicating that PMSCs were metabolized. In addition, in the current research, CD31 and CD34 were highly expressed in vascular endothelium, and there were abundant new capillaries in the tumor. However, there were no significant differences in the range and number of tumor blood vessels between the experimental group and the control group. Ki-67 has been widely regarded as an effective biomarker. Furthermore, it was applied in studies on human subjects or animal models, malignant behavior, aging, and regenerative process. According to past studies, this biomarker can accurately signify the extent and percentage of proliferating cells in various malignancies, including renal cell carcinoma, adenocarcinoma, non–small cell lung cancer, and soft tissue sarcoma, among others. In the current research, Ki-67 was highly expressed in both the experimental and the control group. The ratio of nucleus to cytoplasm was high, which revealed that the increase and decrease of intracellular and extracellular space impacted the role of cancer cell proliferation, whereas the injection of PMSCs did not significantly affect the proliferation of CRC cells.

This study confirmed that the USPIOs-labeled PMSCs had high labeling efficiency, whereas cell viability, proliferation ability, and apoptotic rate were not markedly influenced. Labeled cells injected into CRC transplanted tumors were studied for 14 days. However, only the inhibitory effect on growth of tumor volume has been noted, whereas no meaningful influence on tumor proliferation and angiogenesis was found. In future studies, it may be possible to increase the number of injected PMSCs or establish a less malignant xenograft tumor model to prolong the rate of tumor development to increase the number of injected PMSCs or establish a less malignant stromal tumors. *Front Immunol*. 2015;6:560.

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