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Research article

Early diagnostic method for sepsis based on neutrophil MR imaging

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Abstract

Purpose: This paper aims to evaluate in vitro the labelling efficiency of normal mouse and human neutrophils and neutrophils under sepsis analog conditions with two kinds of superparamagnetic iron oxide nanoparticles (SPIONs) and explore their detection and quantification by MRI at 3T.
Materials and methods: Freshly isolated mouse and human neutrophils were treated with LPS at a concentration of 10 ng/mL as neutrophils under sepsis analog condition. Then control neutrophils and sepsis analog neutrophils were respectively labelled with iron particles in a diameter of 40 nm and 200 nm. The derived four kind of neutrophils were cultured in medium for 1 h. The labelling efficiency was determined with Prussian blue staining and magnetic resonance imaging (3T) for presence of intracellular iron. Data were expressed as the mean ± SD and Student *t* test was used to test statistically significant differences.
Results: Labelled with mannan-coated SPION, both LPS-treated mouse and human neutrophils showed higher iron uptakes by Prussian blue staining and higher T2 signal loss than control group. When labelled with Dextran-coated SPION (Feridex), both mouse and human control and sepsis analog neutrophils displayed lower iron uptake by Prussian blue stain and slighter T2 signal loss.
Conclusion: Mouse and human neutrophils could be more effectively labelled by Mannan-coated SPION in vitro than Feridex. Sepsis analog neutrophils labelled by Mannan-coated SPIONs could be efficiently detected on MR images, which may serve as an early diagnostic method for sepsis.

Keywords: Neutrophils; Cell labelling; SPION; MR imaging

1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome of infectious origins, which is a leading cause of death of critically ill patients. Besides, the mortality rate could be further aggravated by delay in diagnosis and initiation of antibiotics [1].

Current diagnosis of sepsis relies heavily on blood bacteria culture, biomarkers, and clinical manifestations. However, the methods of diagnosis of sepsis are not accurate enough for clinicians to dispense with clinical judgments [2].

The newly developed contrast agents such as particles of super-paramagnetic iron oxide nanoparticle (SPION) have been extended to magnetic resonance (MR) imaging and cellular imaging [3]. Besides, labeling of stimulated (in macrophage medium or endotoxin activated) and control human monocytes with SPIONs presents uptake of iron particles [4,5]. Meanwhile, some coating materials are able to target some specific receptors, such as dextran vs. scavenger receptor and Mannan vs. mannose receptor. Since Mannan, the component on the cell wall of microorganism, could be recognized by mannose receptor of phagocytes, Mannan-coated SPION has been reported used as the MR contrast agent for macrophage targeting [6].

It is known that neutrophils are the first to intervene during an inflammatory process and the key effectors of the innate immune response. It is assumed that use of Mannan-coated

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SPION to target mannose receptors on the surface of neutrophils might offer an earliest way to diagnosis of sepsis.

This study undertook to evaluate the phagocytic effect of neutrophil and the feasibility and diagnostic efficiency of Mannan-coated SPIONs in sepsis models in vitro.

2. Materials and methods

2.1. Isolation of neutrophil from mouse bone marrow

10 5–8-week aged male Balb/c mice were used for the experiment, with approximately 4–6 $\times$ 10^7 neutrophils isolated from each 5 mice. Isolation protocols had been approved by the Institutional Animal Care and Use Committee at the Chonnam National University Hospital of South Korea. Mice neutrophils were isolated from bone marrow cell suspensions, with the femur and tibia flushed with 5 ml RPMI (Roswell Park Memorial Institute medium; Wel GENE Inc, Korea) 1640/penicillin/streptomycin. Then the cells passed through a glass wool column and were pelleted by centrifugation at 1000 rpm for 10 min. The cell pellets were re-suspended in 0.3% fetal calf serum/phosphate-buffered saline (PBS). And then, neutrophils from bone marrow cell suspension were isolated by plasma-percoll gradients after dextran sedimentation of erythrocytes. Isolated neutrophils were counted with hemocytometer and suspended in RPMI media containing 5% FBS (5×10^6 cells/ml). Neutrophil isolation was >97% as determined by Wright–Giemsa-stained cytospin preparations. Cell viability, as determined by trypan blue exclusion, was consistently >98%.

2.2. Isolation of neutrophil from human blood

For human neutrophil isolation, 25 ml peripheral blood was obtained from each 5 healthy volunteers (age <60 years) under a protocol approved by Chonnam National University Hospital's Institutional Review Board, and written informed consent was obtained. Neutrophils were isolated by plasma-percoll gradients after dextran sedimentation of erythrocytes, as described previously [7] 25 ml whole-blood was centrifuged at 1200 rpm for 20 min at room temperature, and the upper layer consisting of plasma and platelets were removed. The platelet rich plasma (PRP) was then transferred to a new tube, with 5 ml dextran added to a final concentration of 6%. Erythrocytes were sedimented under gravity for 45 min at room temperature. The erythrocyte-depleted supernatant was centrifuged at 1100 rpm for 6 min. The resulting leukocyte-enriched pellet was re-suspended in platelet poor plasma (PPP). The leukocytes were then layered over a discontinuous gradient of percoll (of 42% 2 ml and 51% 2 ml) in a 15 ml polypropylene centrifugation tube. The percoll densities were created from stock percoll diluted with PPP. The percoll densities and layered cells were then centrifuged at 1100 rpm for 10 min. Neutrophils were found at the 51–42% percoll layer interface. Moreover, if RBCs stayed with neutrophil, red blood cell lysing buffer (SIGMA, UK) might be applied to lyse the RBC. Neutrophils were removed from the new tube and washed twice with phosphate buffered saline (PBS). Cells were counted with a hemocytometer (Marienfeld, Germany) and re-suspended at 5 $\times$ 10^6 cells/ml with RPMI-1640 contained 5% of serum. The purity (Wright's stain) and viability (trypan blue exclusion) of freshly isolated cells were routinely no less than 97% and 95%, respectively.

2.3. Neutrophil activation

Control neutrophils were divided into two groups. One control group was treated with 10 ng/ml LPS (Lipopolysaccharide) for 2 h to create the sepsis condition. LPS, a major component of the outermost membrane of G (−) bacteria [8], is the key mediator in the development of sepsis caused by G (−) bacteria [9].

2.4. Experimental procedure

The procedures were as follows. 4–5 $\times$ 10^7 neutrophils were harvested from control and sepsis analog group and transferred to 6-well cell culture plates, with each well containing about 1 $\times$ 10^7 neutrophils. Control neutrophils were incubated 2 h in 1 ml RPMI-1640 supplemented with 10% FBS (fetal bovine serum), and penicillin (100 IU/ml)/streptomycin (100 μg/ml) at 37 °C in a humidified CO2 5% atmosphere. Neutrophils under sepsis analog condition were incubated in the same condition of the control group and, added with 10 ng/ml LPS (Sigma-Aldrich; St Louis; USA), were stimulated for 2 h.

2.5. Exposure of cell suspensions to MR contrast agents

After activation for 2 h, neutrophils were changed to former RPMI-1640 media with fresh RPMI-1640 1 ml and supplemental 10% fetal bovine serum, and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Then, each group of neutrophils was added with increasing concentrations (up to 5.0 mg Fe/ml) of Mannan-coated SPION or Feridex (AMAG Pharmaceuticals, Cambridge, USA) and incubated at 37 °C in a humidified CO2 5% atmosphere. 1 h later, the neutrophils were transferred to 15 ml tubes and washed 3 times with PBS by sedimentation (5 min, 1200 RPM) in order to screen out unbound particles. Then, the number of neutrophils was counted in a cell counter. 1 $\times$ 10^6 neutrophils were used for Prussian blue stain, and 2.5 $\times$ 10^6 neutrophils transferred to 0.5 ml phantom tubes for MR imaging in vitro.

2.6. Histochemical detection of SPIONs

After incubation with mannan-coated SPION and Dextran-coated SPION for 1 h, four different kinds of cells were fixed with 4% HCHO and cytospots (1 $\times$ 10^6 cells/slide) were prepared on glass slides with air dried. The presence of SPIONs was detected by Prussian blue stain of iron, and cell spots were counterstained with Nuclear Fast Red Solution. Briefly, cell spots were fixed in acetone and incubated with a 1:1 vol/vol mixture of 2% potassium ferrous cyanide (Sigma;

St Louis; USA) and 2 N HCL(Sigma; St Louis; USA) for 20 min. Glass slides were rinsed in distilled water and counterstained with Nuclear Fast Red Solution (Sigma-aldrich; St Louis; USA) for 5 min. Subsequently slides were washed with distilled water and dehydrated by 70% alcohol, 80% alcohol, 90% alcohol, 95% alcohol respectively for one time, and 100% alcohol for two times, with each immersed for minimum one minute. Glass slide was mounted by Permount solution (Fisher Scientific; New Jersey; USA).

2.7. In vitro MR imaging

In this study, we made a 0.5 ml phantom tube for in vitro MR imaging. Firstly, 200 μl gelation gelatin (10%) was added into the button of 0.5 ml phantom tube. Secondly, 2.5×10^6 neutrophils were transferred into 50 μl 4% HCHO and embedded in 50 μl gelatin 10% (1:1), which were placed into the in the middle layer of phantom tube. Thirdly, another 100 μl gelatin were transferred to the top of the tube. Labeling efficiency was determined with T2 weighted images of 3T MRI (TimTrio, Siemens, Germany). The following MR imaging data were collected, with the field of view at 160 mm × 160 mm, matrix at 189 × 384 and flip angel at 150°. T2 maps were a result of a mono-exponential fit of 10 turbo spin echo MR images with time of repetition and echo (3000 ms, 178 ms; sections of 2 mm thickness). For analysis of relaxation time, regions of interest (circular, 7 mm in diameter) were placed in the center of phantom tube, and special care was taken to exclude areas with air-susceptibility artifacts. Quantitative T2 values were obtained by averaging the middle five regions of interest in the phantom volume. In this way, the most stable values for relaxation time was obtained, with minimum noise levels. Analysis was performed by experienced MR technologists (Heo SU, with 4 years of experience, respectively).

2.8. Statistical analysis

Intracellular iron uptake on T2 weighted signal intensity rates in human neutrophils were expressed as mean ± SD and two-way analysis of variance were analyzed by Student *t* tests. $P < 0.05$ was considered with statistically significant difference.

3. Results

3.1. In vitro MR imaging: mouse neutrophil

T2 images of neutrophils in a phantom tube (Fig. 1) suggested that signal intensity loss was different between the control and LPS-treated neutrophils labeled with Mannan-coated SPION as well as between those labeled by mannan-coated SPION and Dextran-coated SPION.

MR T2 signal intensities were respectively 196 ± 13.6 and 95 ± 1.4 for mouse control neutrophils incubated with mannan-coated SPION (Fig. 1A), and LPS-treated mouse neutrophils (Fig. 1B). T2 signal intensities were respectively 989 ± 35.4 and 1014 ± 57.2 for control neutrophils labeled with Dextran-coated SPION (Fig. 1C) and LPS-treated neutrophils labeled with Dextran-coated SPION (Fig. 1D).

3.2. Histochemical detection of SPIONs in the mouse neutrophils

Control neutrophil presented without positive staining for irons. 5–10% neutrophils of the control group were labelled with Mannan-coated SPION presented with approximately intracellular irons (Fig. 2A). In contrast, 80–90% LPS-treated neutrophils incubated with Mannan-coated SPIONs showed intracellular iron (Fig. 2B). However, there was no iron uptake

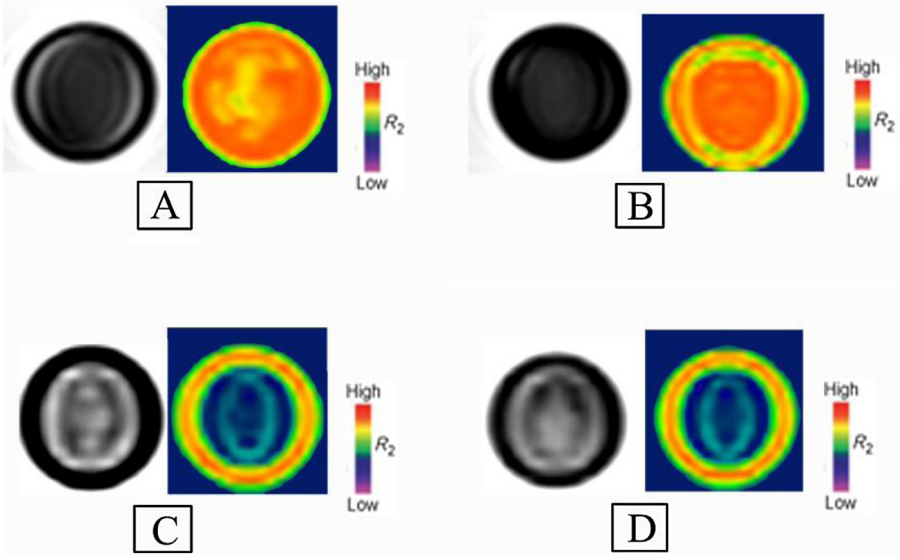

Fig. 1. T_2 weighted images of phantoms containing SPIO agents. (A) control mouse neutrophil incubated with Mannan-coated SPION; (B) LPS-Treated mouse neutrophil incubated with Mannan-coated SPION; (C,D) control and LPS-treated neutrophil incubated with Feridex.

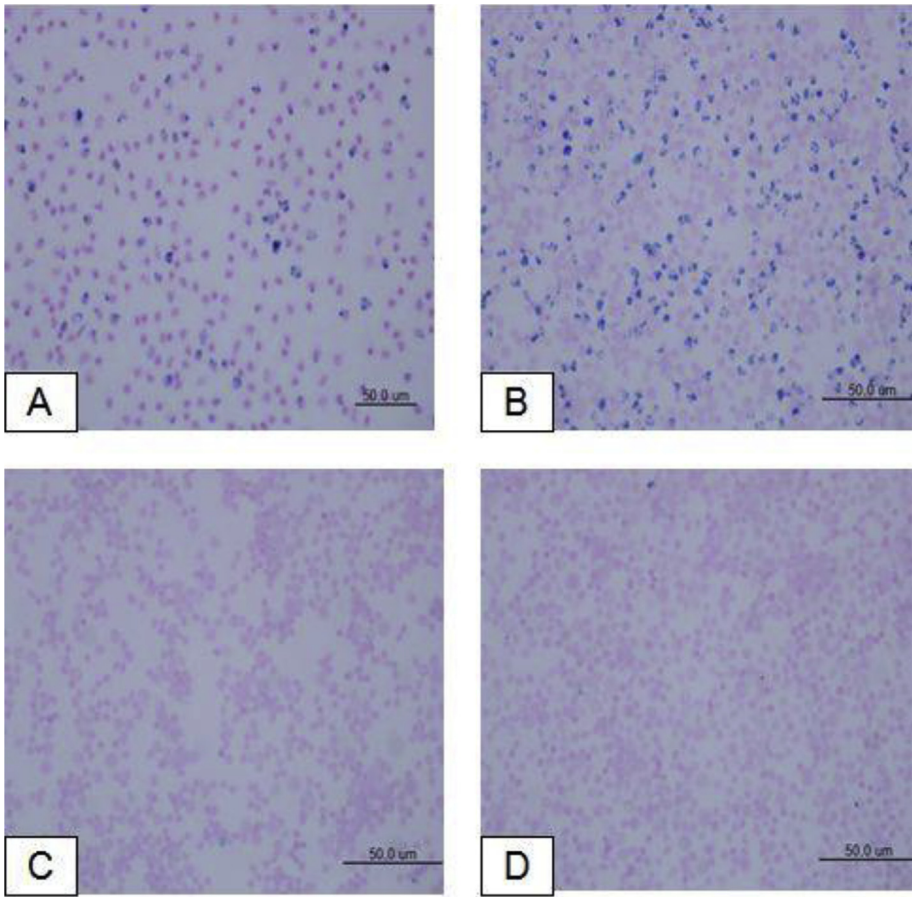

Fig. 2. Iron oxides detected in human neutrophils with Prussian blue staining. Iron oxide is marked in blue, and cell nuclei are in red. (A) control mouse neutrophils incubated with Mannan-coated SPION at a concentration of 5.0 mg Fe/Ml; (B) LPS-treated mouse neutrophil incubated with Mannan-coated SPION; (C, D) control and LPS-treated neutrophil incubated with Dextran-coated SPION at a concentration of 11.2 mg Fe/ml. (Prussian blue stain:original magnification *n*, ×40).

both in control and LPS-treated neutrophils labelled in the Dextran-coated SPION (Fig. 2C&D).

3.3. In vitro MR imaging: human neutrophil

MR T2 mean signal intensities (Fig. 3A) were 431.2 ± 39.43 for human control neutrophils incubated with Mannan-coated SPION, and 219.4 ± 32.88 (Fig. 3B) for LPS-treated human neutrophils labeled with Mannan-coated SPION; while were 1018.8 ± 59.32 for control neutrophils(Fig. 3C) labelled with Dextran-coated SPION and 984.2 ± 50.94 for LPS-treated neutrophils labeled with Dextran-coated SPION(Fig. 3D). As indicated in Fig. 5, both in mouse and human, neutrophils under sepsis-analog condition displayed lower T2 signal intensities than the control, suggesting uptakes of more iron particles in the former.

3.4. Histochemical detection of SPIONs in the human neutrophils

Approximately 5–10% control neutrophils labelled with Mannan-coated SPION showed intracellular irons (Fig. 4A). In contrast, 70–80% LPS-treated neutrophils labeled with Mannan-coated SPIONs presented with intracellular irons in the cytosol (Fig. 4B). Besides, there was barely no iron uptake in both in control and LPS-treated neutrophils labelled with Dextran-coated SPION (Feridex) (Fig. 4C&D).

4. Discussion

The findings suggest that neutrophils were labeled more efficiently by Mannan-coated SPION than Dextran-coated SPION (Feridex). Moreover, neutrophils under sepsis-analog conditions demonstrated with more iron uptakes than control neutrophils.

Stephan Metz et al. has validated use of MR iron oxide contrast agents to evaluate the capacity of human monocytes in phagocytosis [5]. Lee SM et al. remarked that SPIO-enhanced MRI might help evaluate infectious diseases of the joint or soft tissues in light of the uptake of iron particles in fibroblasts as well as macrophage [10].

Like monocyte and macrophage, neutrophil is also a kind of phagocyte. Raoul D.O et al. [11] mentioned that SPION was more effective in labelling human monocyte, and that cellular iron uptake was significantly higher after incubation with SPION compared with that with USPION. Earlier reports

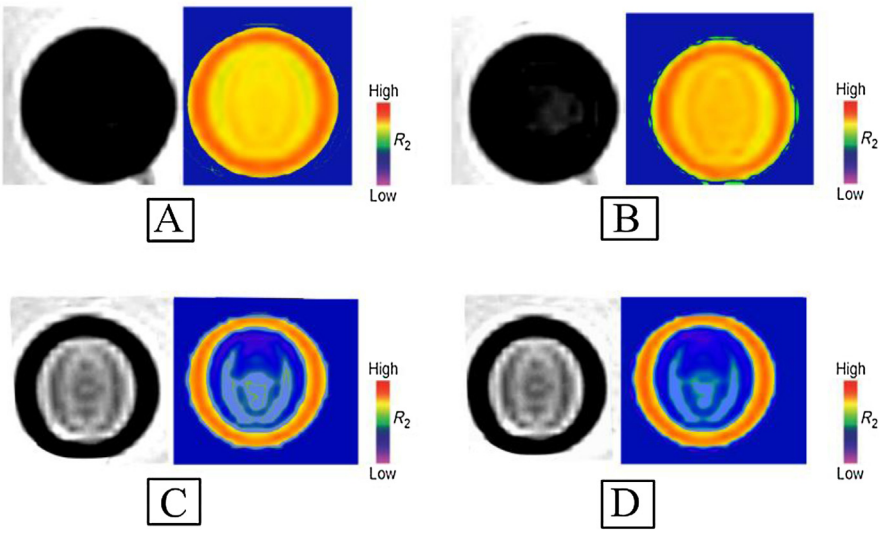

Fig. 3. T_2 weighted images of phantoms containing SPIO agents. (A) control human neutrophil incubated with Mannan-coated SPION; (B) LPS-treated human neutrophil incubated with Mannan-coated SPION; (C, D) control and LPS-treated neutrophil incubated with Feridex.

identified that phagocytotic uptake of iron oxide increased along with particle size [12,13].

However, different from the earlier reports, this study revealed that neutrophil uptake with USPION was higher than SPION, which was confirmed in both mouse and human neutrophils. Higher uptake with USPIONs (diameter 40 nm) than that with SPIONs (diameter 150 nm) in neutrophils might be attributable to following reasons.

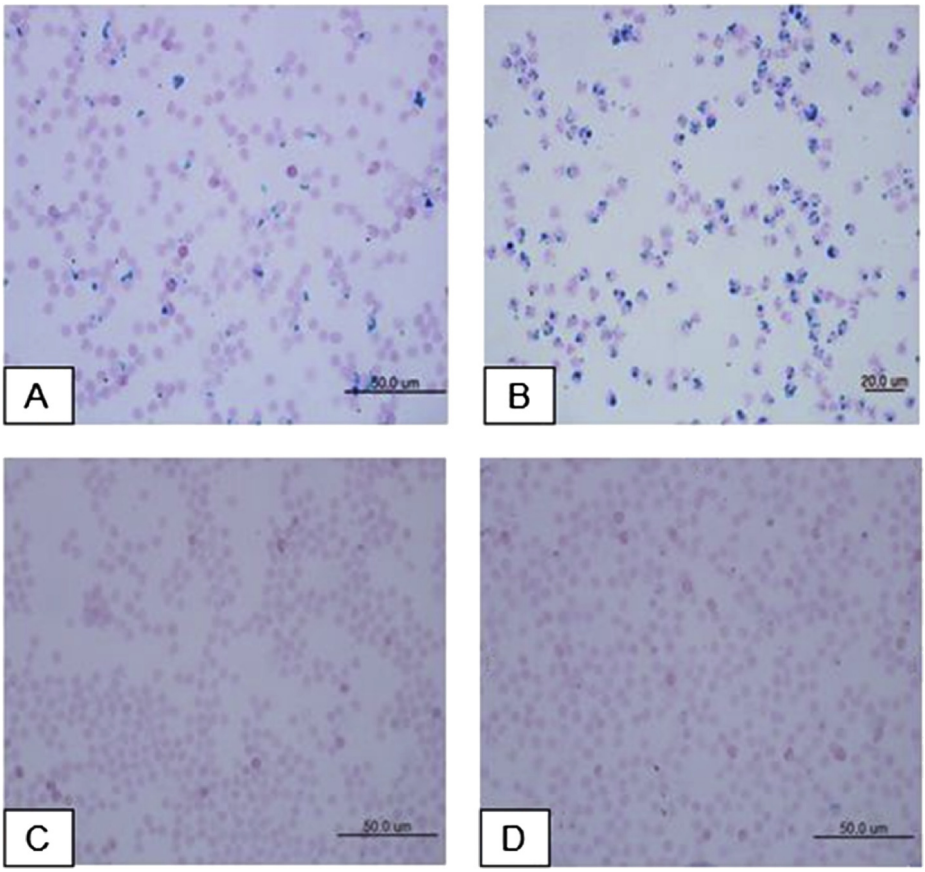

Fig. 4. Iron oxides detected in human neutrophils with Prussian blue staining. Iron oxide is marked in blue, and cell nuclei are in red. (A) control human neutrophil incubated with Mannan-coated SPION; (B) LPS-treated human neutrophil incubated with Mannan-coated SPION; (C, D) control and LPS-treated neutrophil incubated with Feridex. (Prussian blue stain:original magnification, ×40).

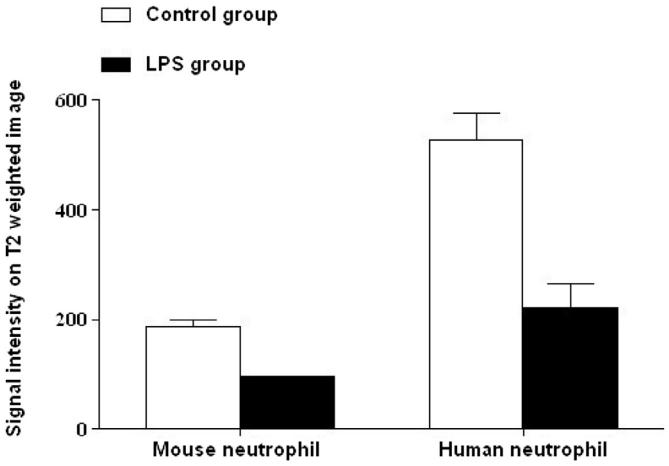

Fig. 5. Graph shows T2 weighted signal of labelled mouse and human neutrophil 1 h after incubation with mannan-coated SPION at a concentration of 5.0 mg Fe/ml.

As a kind of phagocytes, neutrophils have mannose receptors [14]. Therefore mannan-coated SPIONs are easily to combine with neutrophils and such receptor combination reaction might be powerful than the size effect. Meanwhile, the diameter of the neutrophil is smaller than monocyte, respectively 10–12 μm vs. 10–30 μm. And it might be due to the weaker phagocytosis of the neutrophils than monocytes that predisposes neutrophils to phagocytose smaller particles. Moreover, in human blood pool, USPIOs present with a half-life of more than 24 h [15], whereas SPIOs appear with a half-life of no more than 6 min [16], which then limits their possibility to label endogenous neutrophils in circulation.

In addition, Prussian blue staining and MR imaging validated that compared with control neutrophils, LPS-treated neutrophils under sepsis analog condition showed a higher concentration of irons, especially when incubated with Mannan-coated SPION. Mouse neutrophils under sepsis conditions incubated with Mannan-coated SPION were labelled the more efficiently than other kinds of neutrophils labelled in Feridex. Similar results were also documented for human neutrophil, which echoed to literatures about LPS-treated neutrophils. With the presence of LPS 10 ng/mL, neutrophils could be maximally activated after a comparable delay, thus boasting accelerated production of IL-8 and increased phagocytic activity compared with their inactivated counterparts.

Recent studies suggest that neutrophil apoptosis is decreased in systemic inflammatory response syndrome (SIRS) [17,18], sepsis, and ARDS. Besides some studies indicate that neutrophil apoptosis may serve as a marker of the severity of sepsis [19]. Felix MK et al. even found that superparamagnetically labelled neutrophils could act as abscess-specific contrast agent for MRI [20]. Therefore, MR imaging of neutrophils under sepsis condition and apoptotic neutrophils with Mannan-coated SPION may provide an early diagnostic method for sepsis.

5. Conclusion

This study labelled control neutrophils and with two different kinds of SPIONs (Mannan coated SPION and Dextran-coated SPION). Prussian blue staining and MR imaging showed that neutrophils under sepsis-analog condition contain higher iron particles and so do neutrophils labelled by Mannan-coated SPIONs. MR imaging of neutrophils labelled with Mannan-coated SPION may offer an early diagnostic method of sepsis.

References

[1] Dombrovskiy VY, Martin AA, Sunderram J, Paz HL. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. Crit Care Med 2007 May; 35(5):1244–50.
[2] Kibe S, Adams K, Barlow G. Diagnostic and prognostic biomarkers of sepsis in critical care. J Antimicrob Chemother 2011 Apr;66(Suppl 2):ii33–40. http://dx.doi.org/10.1093/jac/dkq523.
[3] Weissleder R, Elizondo G, Wittenberg J, Rabito CA, Benqele HH, Josephson L. Ultrasmall superparamagnetic iron oxide: characterization of a new class of contrast agents for MR imaging. Radiology 1990 May;175(2):489–93.
[4] Zelivyanskaya ML, Nelson JA, Poluektova L, Uberti M, Mellon M, Gendelman HE, et al. Tracking superparamagnetic iron oxide labeled monocytes in brain by high-field magnetic resonance imaging. J Neurosci Res 2003 Aug 1;73(3):284–95.
[5] Metz S, Bonaterra G, Rudelius M, Settles M, Rummeny EJ, Daldruplink HE. Capacity of human monocytes to phagocytose approved iron oxide MR contrast agents in vitro. Eur Radiol 2004 Oct;14(10):1851–8. Epub 2004 Jul 13.
[6] Yoo MK, Park IY, Kim IY, Park IK, Kwon JS, Jeong HJ, et al. Superparamagnetic iron oxide nanoparticles coated with Mannnan for macrophage targeting. J Nanotechnol 2008 Oct;8(10):5196–202.
[7] Kwak SH, Mitra S, Bdeir K, Strassheim D, Park JS, Kim JY, et al. The kringle domain of okinase-typeplasminogen actvator potentiates LPS-induced neutrophil activation through interaction with {alpha}V {beta}3 integrins. J Leukoc Biol 2005 Oct;78(4):937–45. Epub 2005 Jul 20.
[8] Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol Rev 1993;57(3):655–82.
[9] Suffredini AF, Fromm RE, Parker MM, Brenner M, Kovacs JA, Wesley RA, et al. The cardiovascular response of normal humans to the administration of endotoxin. N Engl J Med 1989 Aug 3;321(5):280–7.
[10] Lee SM, Lee SH, Kang HY, Baek SY, Kim SM, Shin MJ. Assessment of musculoskeletal infection in rats to determine usefulness of SPIO-enhanced MRI. AJR Am J Roentgenol 2007 Sep;189(3):542–8.
[11] Oude Engberink RD, van der Pol SM, Dopp EA, de Vries HE, Blezer EL. Comparison of SPIO and USPIO for in vitro labeling of human monocytes: MR detection and cell function. Radiology 2007 May; 243(2):467–74.
[12] Daldrup-Link HE, Rudelius M, Oostendorp RA, Settles M, Piontek G, Metz S, et al. Targeting of hematopoietic progenitor cells with MR contrast agents. Radiology 2003 Sep;228(3):760–7. Epub 2003 Jul 24.
[13] Matuszewski L, Persigehl T, Wall A, Schwindt W, Tombach B, Fobker M, et al. Cell tagging with clinically approved iron oxides: feasibility and effect of lipofection, particle size, and surface coating on labeling efficiency. Radiology 2005 Apr;235(1):155–61. Epub 2005 Mar 4.
[14] Hall SE, Savill JS, Henson PM, Haslett C. Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a Mannose/Fucose-specific lectin. J Immunol 1994 Oct 1;153(7):3218–27.

[15] McLachlan SJ, Morris MR, Lucas MA, Fisco RA, Eakin MN, Fowler DR, et al. Phase I clinical evaluation of a new iron oxide MR contrast agent. J Magn Reson Imaging 1994 May−Jun; 4(3):301−7.

[16] Weissleder R, Stark DD, Engelstad BL, Bacon BR, Compton CC, White DL, et al. Superparamagnetic iron oxide: pharm acoki netics and toxicity. AJR Am J Roentgenol 1989 Jan;152(1):167−73.

[17] Papathanassoglou ED, Moynihan JA, McDermott MP, Ackerman MH. Expression of Fas (CD95) and Fas ligand on peripheral blood mononuclear cells in critical illness and association with multiorgan dysfunction severity and survival. Crit Care Med 2001 Apr; 29(4):709−18.

[18] Jimenez MF, Watson RW, Parodo J, Evans D, Foster D, Steinberg M, et al. Dysregulated expression of neutrophil apoptosis in the systemic inflammatory response syndrome. Arch Surg 1997 Dec;132(12):1263−9. discussion 1269−1270.

[19] Fialkow L, Fochesatto Filho L, Bozzetti MC, Milani AR, Rodriques Filho RM, Ladniuk RM, et al. Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome. Crit Care 2006;10(6):R155.

[20] Krieg FM, Andres RY, Wintehalter KH. Superparamagnetically labelled neutrophils as potential abscess-specific contrast agent for MRI. Magn Reson Imaging 1995;13(3):393−400.