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# Transferrin receptor and ferritin-H are developmentally regulated in oligodendrocyte lineage cells<sup>☆</sup>

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## Abstract

Iron is an essential trophic element that is required for cell viability and differentiation, especially in oligodendrocytes, which consume relatively high rates of energy to produce myelin. Multiple iron metabolism proteins are expressed in the brain including transferrin receptor and ferritin-H. However, it is still unknown whether they are developmentally regulated in oligodendrocyte lineage cells for myelination. Here, using an *in vitro* cultured differentiation model of oligodendrocytes, we found that both transferrin receptor and ferritin-H are significantly upregulated during oligodendrocyte maturation, implying the essential role of iron in the development of oligodendrocytes. Additional different doses of Fe<sup>3+</sup> in the cultured medium did not affect oligodendrocyte precursor cell maturation or ferritin-H expression but decreased the expression of the transferrin receptor. These results indicate that upregulation of both transferrin receptor and ferritin-H contributes to maturation and myelination of oligodendrocyte precursor cells.

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## Key Words

neural regeneration; neurogenesis, oligodendrocyte; iron; transferrin receptor; ferritin-H; development; myelination; proliferation; induced differentiation; grants-supported paper; photographs-containing paper; neuroregeneration

## Research Highlights

- (1) Transferrin receptor and ferritin-H are expressed in oligodendrocyte lineage cells.
- (2) Transferrin receptor and ferritin-H are upregulated during maturation of oligodendrocyte lineage cells.
- (3) Additional iron does not affect oligodendrocyte precursor cell maturation or ferritin-H expression but decreases the expression of transferrin receptor.

## Abbreviations

TfR, transferrin receptor; OPCs, oligodendrocyte precursor cells; F-H, ferritin-H

## INTRODUCTION

Iron is essential for multiple functions in the central nervous system, including DNA synthesis, gene expression, mitochondrial electron transport and myelination<sup>[1]</sup>. Oligodendrocytes stain for iron more

robustly than any other cell in the normal adult brain<sup>[2-3]</sup>. The highest period of iron uptake in the central nervous system coincides with the peak of myelination<sup>[4-5]</sup>. This is consistent with impaired latency of auditory brain stem potentials and visual evoked potentials (indirect markers of myelination) in iron-deficient children<sup>[6]</sup> and

decreased myelin proteins, lipids and cholesterol in iron-deficient animals<sup>[7]</sup>, suggesting a critical role for iron in oligodendrocyte maturation. Because oligodendrocytes cannot synthesize iron, the way in which developing oligodendrocytes acquire iron should be elucidated.

Transferrin receptor (TfR) is the main protein that mediates the entry of iron to cells by binding to holo-transferrin (iron bound transferrin, holo-Tf), which may promote oligodendrocyte precursor cell (OPC) differentiation to a multipolar morphology, together with increased expression of myelin basic protein, myelin associated glycoprotein and O4 and O1 proteins (stage-specific markers of oligodendrocyte development)<sup>[8]</sup>. Surprisingly, several studies have failed to detect TfR in white matter tracts and oligodendrocytes in adult animals by immunohistochemistry and *in situ* hybridization<sup>[9-10]</sup>. Although TfR may be downregulated during cell maturation, oligodendrocytes remain the principal brain cells that stain for iron, which may be attributed to ferritin-H (F-H), a high-capacity iron storage protein. Indeed, F-H-deficient mice showed significant decreases in galactolipids, cholesterol, phospholipids, and proteolipid protein in myelin compared with normal mice<sup>[7]</sup>. Recent studies showed that microinjection of ferritin into intact white matter could increase OPC proliferation and promote the generation of oligodendrocytes in rats<sup>[11]</sup>. In addition, iron efflux from astrocytes plays a role in remyelination<sup>[12]</sup>, suggesting that iron may directly or indirectly regulate oligodendrocyte lineage cell development. Although TfR and F-H are essential for oligodendrocyte development, it is still unknown whether they are developmentally regulated. To determine whether TfR and F-H are developmentally regulated during maturation of oligodendrocyte lineage cells, we examined TfR and F-H expression by virtue of an *in vitro* induced differentiation model of purified OPCs. We also detected whether additional iron could affect OPC differentiation and the expression of TfR and F-H.

## RESULTS

### Purified OPC culture from P0 rat cerebral cortex

We obtained a highly purified OPC culture under proliferation culture conditions. As shown in Figure 1, more than 95% of the cells in culture were A2B5<sup>+</sup> and NG2<sup>+</sup> (markers for oligodendrocyte precursor cells<sup>[13]</sup>) OPCs exhibited a typical bipolar morphology.

### OPC differentiation *in vitro* by triiodothyronine/thyroxine (T3/T4) induction

When B104 conditioned medium<sup>[14]</sup> was withdrawn from

the culture medium, and 30 ng/mL T3/40 ng/mL T4 were added into cultured medium (standard protocol to induce differentiation of oligodendrocytes), OPCs gradually developed more complex branches and differentiated to immature or mature oligodendrocytes that expressed myelin basic protein (a marker for matured oligodendrocytes<sup>[15]</sup>) after 3–4 days of growth (Figure 2). Myelin basic protein was also significantly increased in oligodendrocytes cultured in differentiation medium compared with proliferative medium as measured by western blot ( $P < 0.01$ ).

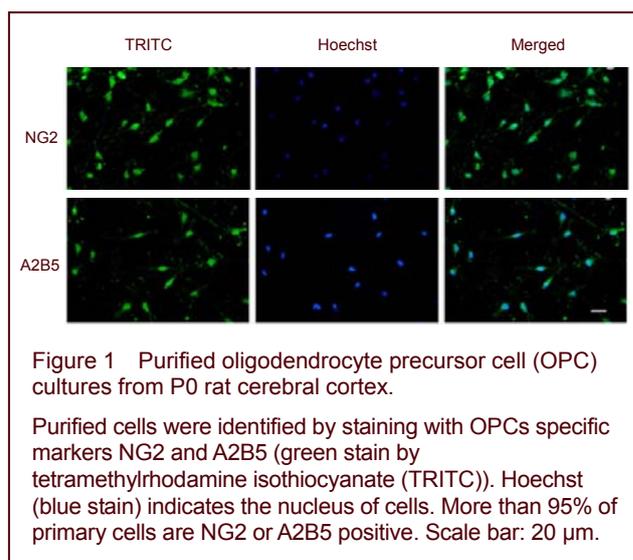


Figure 1 Purified oligodendrocyte precursor cell (OPC) cultures from P0 rat cerebral cortex.

Purified cells were identified by staining with OPCs specific markers NG2 and A2B5 (green stain by tetramethylrhodamine isothiocyanate (TRITC)). Hoechst (blue stain) indicates the nucleus of cells. More than 95% of primary cells are NG2 or A2B5 positive. Scale bar: 20  $\mu$ m.

### Both TfR and F-H increased during maturation of oligodendrocytes

OPCs were differentiated in culture medium for 3–4 days and subjected to western blot assay. Results showed that both TfR and F-H were significantly increased when cells were grown in differentiation medium compared with proliferative medium (Figure 3), which further confirmed the importance of iron in the maturation of oligodendrocytes.

### Additional iron did not affect OPC maturation but decreased the expression of TfR

To further understand whether additional iron could affect the expression of TfR and F-H during the maturation of OPCs, we added different doses of Fe<sup>3+</sup> (free iron, which binds to apo-transferrin to form holo-transferrin) from 5–50 ng/mL (higher doses of Fe<sup>3+</sup> are toxic) into the cultured medium for 4 days. We observed that additional iron did not affect OPC maturation (Figure 4A). However, additional iron decreased the expression of TfR during OPC maturation ( $P < 0.05$ ), which was similar to other cellular responses to iron overload<sup>[16]</sup>, but did not affect F-H expression (Figure 4B).

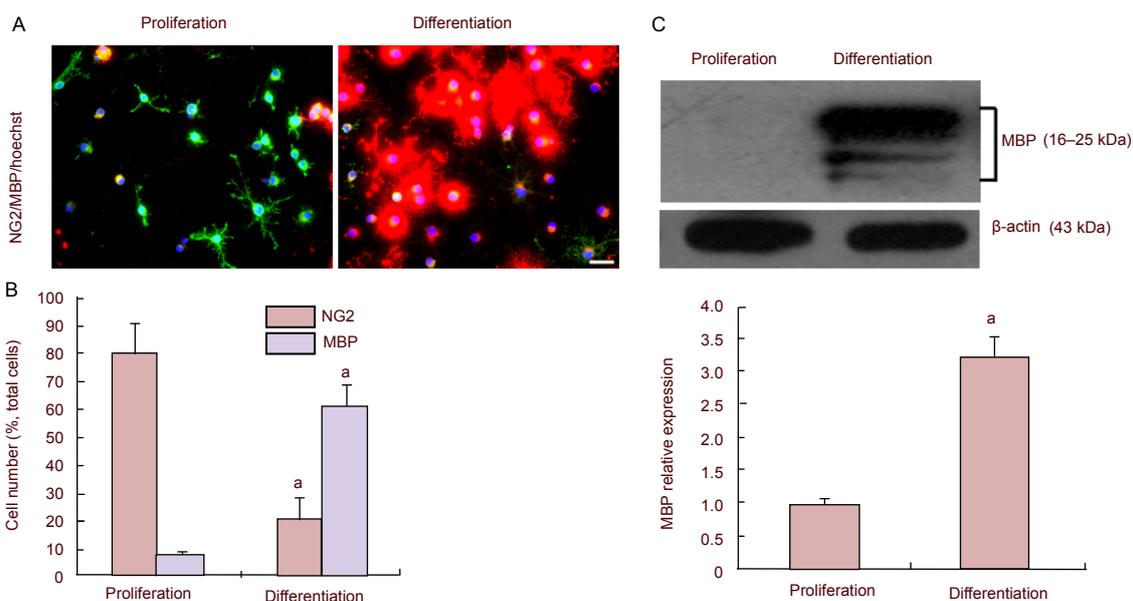


Figure 2 Oligodendrocyte precursor cell (OPC) differentiation by 30 ng/mL triiodothyronine/40 ng/mL thyroxine induction *in vitro*.

(A) Representative immunostaining showed maturation of OPCs when cultured in proliferation or differentiation medium for 3 days, respectively. NG2-labeled OPCs (green cells) and myelin basic protein (MBP) stained matured oligodendrocytes (red cells) were observed. Scale bar: 20  $\mu$ m.

(B) Statistical data for NG2/MBP<sup>+</sup> cells proportions in cultures grown with different media. <sup>a</sup> $P < 0.01$ , vs. proliferation medium.

(C) Western blots showed that MBP was significantly increased in cells grown in differentiation medium for 3 days compared with cells grown in proliferation medium. Expression of MBP is expressed by absorbance ratio of MBP against  $\beta$ -actin, and then normalized as relative fold to controls. Data are shown as mean  $\pm$  SEM. <sup>a</sup> $P < 0.01$ , vs. proliferation medium (two-tailed student's *t*-test).

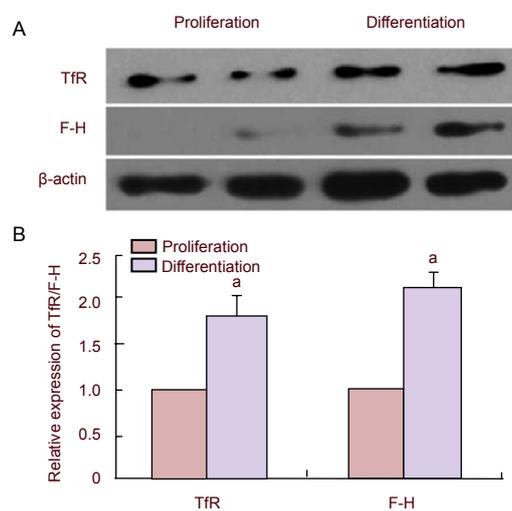


Figure 3 Both transferrin receptor (TfR) and ferritin-H (F-H) increase during maturation of oligodendrocytes.

(A) Representative blots of TfR (95 kDa) and F-H (20 kDa) showed that TfR and F-H were significantly increased in cells grown in differentiation medium for 3 days compared with those grown on proliferation medium.

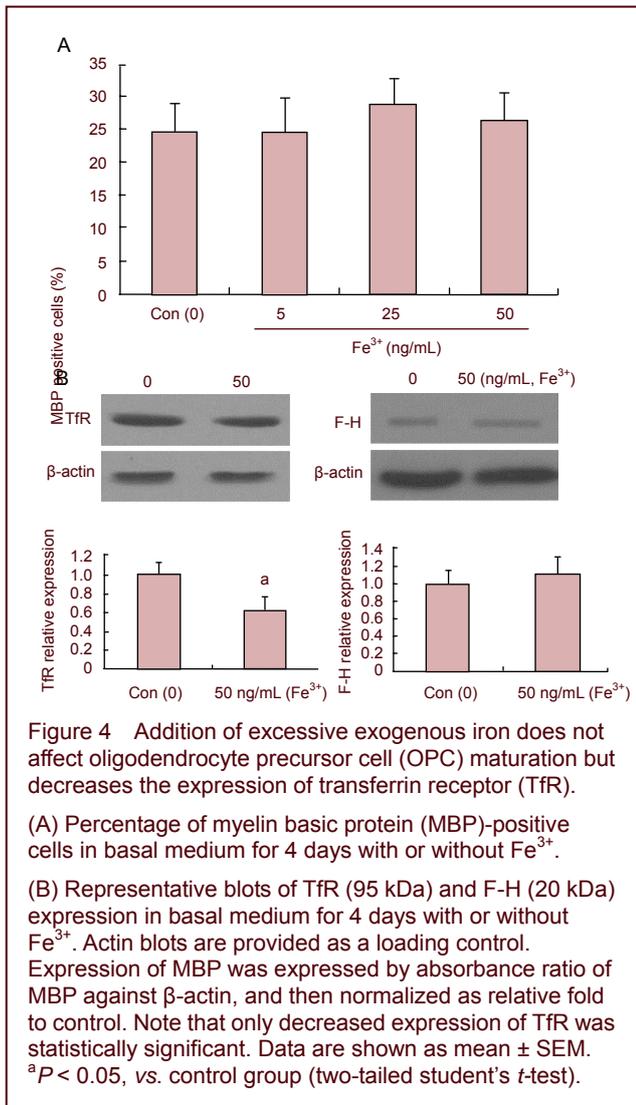
(B) Statistical data from three independent blots. Expression of TfR and F-H were expressed by absorbance ratio of TfR and F-H against  $\beta$ -actin, and then normalized as relative fold to controls. Data are shown as mean  $\pm$  SEM. <sup>a</sup> $P < 0.05$ , vs. proliferation medium (two-tailed student's *t*-test).

## DISCUSSION

Oligodendrocytes are the only cells in the brain involved in myelin production and are the principal cells in the brain that stain for iron<sup>[17]</sup>. Compelling evidence for the importance of iron in myelin production is provided by human and animal studies that demonstrated severe chronic iron deficiency causes hypomyelination<sup>[18]</sup>. Thus, iron is an essential factor for oligodendrocyte survival and function. However, a complete understanding of the molecular mechanisms of iron entry into oligodendrocytes is a prerequisite to understand oligodendrocyte development and develop effective therapeutic strategies for hypomyelination.

This study shows that both TfR and F-H are developmentally upregulated in oligodendrocyte lineage cells, which implies an essential role for iron in the maturation of oligodendrocytes and myelinogenesis. Previous reports showed the significance of iron in oligodendrocyte lineage cell development as chronic severe iron deficiency caused hypomyelination<sup>[19]</sup>. Although a requirement of iron for myelin production is generally accepted, it is not clear whether iron deficiency

leads to global brain hypomyelination by affecting oligodendrocyte numbers or their differentiation state or both<sup>[20]</sup>.



TfR and F-H are the main iron metabolism proteins and control iron entry to cells, and iron store and transfer, respectively. Previous studies focused on the iron delivery protein, transferrin, but not on the receptor expressed on oligodendrocytes. Developing oligodendrocytes express TfR as identified by binding assay<sup>[21]</sup> and immunocytochemistry<sup>[22]</sup>. However, TfR levels were reduced in younger animals during development and were not detected in the white matter of adult humans or rodents<sup>[23]</sup>. In contrast, we demonstrated here that TfR was expressed in OPCs and significantly increased when cells differentially matured. Our findings may be because of different characteristics of our *in vitro* culture system, or other unknown mechanism(s). In addition, we also expected that the time from oligodendrocyte maturation to myelinogenesis would be a key transition for the expression of iron

metabolism proteins; that is, the loss of TfR would occur during myelinogenesis but not in the period of oligodendrocyte differentiation. Accumulating evidence suggests that there is an alternative pathway for iron entry to oligodendrocytes. Furthermore, this pathway involves F-H that becomes dominant as animals age, and a similar system is present in humans<sup>[24-25]</sup>. Consistent with our observations, F-H is upregulated during the maturation of oligodendrocytes.

Although iron is essential for oligodendrocyte maturation, additional free iron (Fe<sup>3+</sup>) does not affect OPC maturation. One reason could be the negative feedback of TfR on iron overload. Our data showed that additional iron could downregulate TfR expression during OPC maturation, resulting in limited absorption of iron into OPCs. Recently, Todorich *et al*<sup>[26]</sup> found that the addition of extracellular F-H and membrane permeable 3,5,5-trimethylhexanoyl ferrocene stimulated the development of OPCs by increasing myelin basic protein expression but not membrane-impermeable ferric ammonium citrate. However, apo-transferrin (Fe<sup>3+</sup> free transferrin, which binds to Fe<sup>3+</sup> to form holo-transferrin and imports iron into cells) promotes OPC maturation in culture systems *in vitro* or *in vivo* development and injury models<sup>[27]</sup>. In summary, iron imported into oligodendrocytes by indistinct mechanisms can upregulate both TfR and T-H and is critical for OPC maturation.

## MATERIALS AND METHODS

### Design

A controlled, cytobiological experiment.

### Time and setting

The experiment was performed at Central Laboratory in Shanghai Tongji Hospital, Tongji University School of Medicine, China from January 2011 to January 2012.

### Materials

A total of 20 clean, healthy, newborn Sprague-Dawley rats were purchased from the Chinese Academy of Medical Sciences, China (license No. SCXK (Hu) 2008-0016). All experimental protocols were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China<sup>[28]</sup>.

### Methods

#### Rat primary oligodendrocyte lineage cell culture

Purified OPCs were isolated using the shake-off method

as previously described with some modifications<sup>[29]</sup>. Briefly, newborn (postnatal day 1) rats were decapitated and mixed cortical glial cell cultures were generated from cerebra and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (D10) for 10 days in 75 cm<sup>2</sup> flasks at 37°C and 5% CO<sub>2</sub> with a medium change every 3 days. Microglia were roughly separated by shaking the culture flasks for 1 hour at 250 r/min on a gyratory shaker at 37°C. Cells were rinsed three times with PBS to remove the detached microglia. The culture flasks were further shaken for an additional 16 hours at 280–290 r/min at 37°C, to separate the OPCs. To remove contaminating microglia and astrocytes in the isolated OPC cultures, the detached cell suspension was seeded and left to adhere twice in uncoated Petri dishes (Sterilin, Staffordshire, UK) for 2 hours at 37°C with 5% CO<sub>2</sub>. The collected OPCs in D10 medium were then re-plated at densities between 50 000 and 5 000 cells/cm<sup>2</sup> onto poly-L-lysine-coated 24-well plates, dishes, or coverslips and maintained at 37°C with 5% CO<sub>2</sub>. Finally, to remove any remaining microglia, 6 hours after plating OPC cultures were treated with 5 mM leucine methylester (Sigma, St. Louis, MO, USA) for 15 minutes at room temperature and were then washed three times with DMEM<sup>[30]</sup>. Cells were expanded in B104 conditioned medium to further enrich and expand the oligodendrocyte cultures<sup>[14]</sup>. For differentiation assays, 30 ng/mL T3 and 40 ng/mL T4 were added to the medium<sup>[31]</sup>. As the cultures in differentiation medium contained heterogeneous cells that were in different stages including OPCs and mature oligodendrocytes, we adopted the term "oligodendrocyte lineage cells" to define the whole population in the cultures<sup>[32]</sup>.

#### ***Immunofluorescence staining of myelin basic protein, NG2, A2B5, TfR and F-H expression in oligodendrocytes***

Immunocytochemistry of iron-related proteins and oligodendrocyte lineage cells was performed as described previously<sup>[15, 33-34]</sup>. After 3 days of culture in differentiation medium, oligodendrocytes were fixed with 4% paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 5 minutes. Cells were incubated with mouse anti-rat myelin basic protein monoclonal antibodies (1:100; Millipore, Billerica, MA, USA), rabbit anti-NG2 polyclonal antibody (1:200; Chemicon, Temecula, CA, USA) and rabbit anti-A2B5 polyclonal antibody (1:100; Chemicon) overnight at 4°C. Final detection was performed by incubation with FITC conjugated goat anti-rabbit IgG antibodies (Sigma) or TRITC-conjugated

goat anti-mouse IgG antibodies diluted 1:400 in PBS containing 5% normal goat serum for 1 hour at 37°C. Cells were counted after Hoechst33342 nuclear staining (Sigma). An Olympus microscope (IX70) connected to a computer equipped with a charge-coupled device camera (Olympus Corporation, Japan) was used to visualize and capture images, which were then analyzed by image analysis software (MetaMorph version 4, Universal Imaging Corporation, Auburn, WA, USA). For NG2<sup>+</sup> or myelin basic protein<sup>+</sup> cell number analysis, cells from 10 randomly captured fields were analyzed per well of triplicate wells for individual treatments in three independent experiments, counting approximately 3 000 cells for each data point. Mean numbers of NG2<sup>+</sup> or BMP<sup>+</sup> cells per field of view were normalized to controls and the results were expressed as relative to controls.

#### ***Western blot analysis of myelin basic protein, TfR and F-H expression in oligodendrocytes***

For myelin basic protein<sup>[32]</sup>, TfR<sup>[35]</sup> and F-H<sup>[26, 36]</sup> expression analysis, OPCs were allowed to differentiate for 3 days in differentiation medium. Whole-cell lysates were obtained by radioimmune precipitation assay with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM phenylmethyl sulfonylfluoride, 1 × Roche complete mini protease inhibitor) on ice. Lysates were centrifuged at 12 000 × g for 10 minutes at 4°C, and protein concentration in the supernatant was determined by bicinchoninic acid assay (Sigma). After boiling for 5 minutes, 15 µg of total protein from whole-cell lysates was separated by 15% SDS-PAGE denaturing gel and were electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Inc. Dassel, Germany). Membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing Tween-20 (50 mM Tris, 150 mM NaCl, and 0.1% Tween-20, pH 7.4) for 1 hour, and immunoblotted with mouse anti-myelin basic protein monoclonal antibodies (Millipore, Billerica, MA, USA), mouse anti-β-actin monoclonal antibodies (Abcam, Cambridge, MA, USA), rabbit anti-TfR polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit anti-F-H polyclonal antibodies (Santa Cruz Biotechnology) at a dilution of 1:1 000 overnight at 4°C. Horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (1:400; Santa Cruz Biotechnology) were used for immunoblotting to allow the detection of the final signal with enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA). Quantifications of myelin basic protein, TfR and F-H expression were performed by scanning densitometric analysis on myelin basic protein,

TfR and F-H relative to actin levels. Scanning absorbances were normalized and expressed as relative fold to controls. Results were from three independent experiments.

### Statistical analysis

Data are expressed as mean  $\pm$  SEM from at least three independent experiments unless otherwise indicated. Differences between groups were statistically tested by one-way analysis of variance followed by two-tailed *t*-test. A value of  $P < 0.05$  was considered statistically significant.

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**Author contributions:** Yunxia Li was responsible for the funding, study concept and design, experimental analysis, and manuscript writing. Qiang Guan participated in some experimental analysis and statistical processing. Yuhui Chen, Hongjie Han and Wuchao Liu were responsible for the experimental animal breeding and processing, and provided information support. Zhiyu Nie was responsible for the experiment concept and design, validation and guidance of the study. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** This experiment was approved by the Animal Ethics Committee of Shanghai in China.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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