Autophagy Promoted Neural Differentiation of Human Placenta-derived Mesenchymal Stem Cells

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Abstract. Background/Aim: Human placenta-derived mesenchymal stem cells (hPMSCs) are multipotent and possess neurogenicity. Numerous studies have shown that Notch inhibition and DNA demethylation promote neural differentiation. Here, we investigated the modulation of autophagy during neural differentiation of hPMSCs, induced by DAPT and 5-Azagycidine. Materials and Methods: hPMSCs were treated with DAPT to induce neural differentiation, and the autophagy regulating molecules were used to assess the impact of autophagy on neural differentiation. Results: The hPMSCs presented with typical mesenchymal stem cell phenotypes, in which the majority of cells expressed CD73, CD90 and CD105. hPMSCs were multipotent, capable of differentiating into mesodermal cells. After treatment with DAPT, hPMSCs upregulated the expression of neuronal genes including SOX2, Nestin, and βIII-tubulin, and the autophagy genes LC3I/II and Beclin. These genes were further increased when 5-Azagycidine was co-supplemented in the culture medium. The inhibition of autophagy by chloroquine impeded the neural differentiation of hPMSCs, marked by the downregulation of βIII-tubulin, while the activation of autophagy by valproic acid (VPA) instigated the emergence of βIII-tubulin-positive cells. Conclusion: During the differentiation process, autophagy was modulated, implying that autophagy could play a significant role during the differentiation of these cells. The blockage and stimulation of autophagy could either hinder or induce the formation of neural-like cells, respectively. Therefore, the refinement of autophagic activity at an appropriate level might improve the efficiency of stem cell differentiation.

Stem cell technology is promising for manipulating human illnesses in terms of disease modeling, tissue engineering, drug discovery and cell therapy (1). One major issue in embryonic stem cell (ESC) research is safety and the ethical issues surrounding this area. In this regard, many groups have made an attempt to identify and characterize adult stem cells (ASCs) for future therapies (2). The best characterized ASC populations reside in the bone marrow (BM). BM-derived mesenchymal stem cells (MSCs) are considered to be a potential source for stem cell therapies due to their plasticity and potent immunosuppressive capabilities (3). Despite their capacity and potency, the difficulty in obtaining BM aspirates from patients is a problem; hence, alternative sources of stem cells, human placenta tissue has gained increasing attention as an attractive source of adult stem cells, as they are considered medical waste, non-invasive and easy to isolate (4, 5). Human placental-derived mesenchymal stem cells (hPMSCs) contain a great potential to differentiate into various cell types, including neuronal cells (6). Numerous studies have shown success in differentiating hPMSCs towards the neural lineage under specific conditions (7, 8). Therefore, neural differentiation from hPMSCs provides a promising cell-based therapy for neurodegenerative diseases, such as Alzheimer’s, Parkinson’s and Huntington’s disease.

In this study, we focus on the Notch signaling which has a critical role for directing differentiation of stem cells into several cell types (9-11). Notch signaling is activated when Notch receptor binds to its ligand, and results in the cleavage of the Notch intracellular domain (NICD) of the Notch receptor. NICD can then translocate into the nucleus and initiate the transcription of Notch target genes (12, 13). One
of the most significant approaches for directing cell reprogramming is applying the small chemical molecules at various targets of cell regulation pathways. Recent developments in neural differentiation have heightened the need for the inhibition of Notch signaling pathway by γ-secretase inhibitors such as DAPT, which was shown to induce neural differentiation (14-16). In this study, we set out to differentiate harvested hPMSs toward the neural lineage using a combination of small molecules of differentiation protocols originally designed for hPMSCs. There is a solid connection between epigenetic modifiers and the changes in gene expression profiles during stem cell differentiation (17). DNA demethylating agents can inhibit methylation at gene promoters which is possibly a prerequisite for transcription activation. 5-Azacytidine is a commonly used DNA demethylation compound, which can integrate into DNA and inhibit DNA methylation (18, 19). The combination of DAPT and 5-Azacytidine might thus particularly impact neural differentiation of hPMSCs.

Autophagy is a key mechanism involved in cell self-renewal, cell differentiation, and embryonic development, and acts as cellular cleaning process to get rid of invading microorganisms and toxic aggregated proteins. Autophagy is initiated by the formation of double-membrane-bound vesicles, called autophagosomes, which then fuse with lysosomes to enable the degradation of autophagic cargos and the subsequent recycling of nutrients and membranes (20). Recent evidence suggests that autophagy is known as an important event for stem cell differentiation, for instance the differentiation of muscle stem cells, mesenchymal stem cells, and human induced pluripotent stem cell (iPSCs) (21-23). To date, there has been limited explorations the roles of autophagy during neural differentiation of human mesenchymal stem cells using small molecules. As no definitive proof is available, it is substantial to have a clear understanding on the neural differentiation process of hPMSCs by targeting Notch signaling and the modified levels of autophagy. In this study, we induced hPMSCs towards the neural lineage by DAPT and 5-Azacytidine and observed the effect of autophagy modulation during hPMSCs differentiation.

Materials and Methods

Isolation and cultivation of hPMSCs. Human placental tissues were collected from healthy donors with written informed consent from the Suranaree University of Technology Hospital (SUTH, Nakhon Ratchasima, Thailand). The protocol was approved by the ethics committee of Suranaree University of Technology. The human placental tissues were isolated and cultured according to the guidelines from the previous report with some modification (24). Briefly, placental tissue was minced into small pieces and placed in a dish with 4 mg/ml collagenase/dispase (Roche, Germany) to digest at 37°C for 1 h. Digested tissues were maintained in medium containing Dulbecco’s Modified Eagle Medium high glucose (DMEM/HG; HyClone, Logan, UT, USA), supplemented with 20% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 1 mM L-glutamine, 1 mM Minimal essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml Penicillin and 100 g/ml Streptomycin (Sigma-Aldrich). hPMSCs were incubated at 37°C, in 5% CO₂ for 7 to 14 days. The medium was replaced every 3 days until fibroblast-like cells migrated out from the placental tissues. hPMSCs were maintained and passaged when reaching 90-100% confluence, and 3 independent lines of hPMSCs were used for all experiments.

Characterization of hPMSCs. hPMSCs were seeded approximately at a density of 2x10⁴ cells/well on a 6-well culture plate (Nunc, Roskilde, Denmark), coated with Geltrex basement membrane (Gibco). For osteogenic induction, hPMSCs were cultured in osteogenic induction medium, consisting of DMEM, 10% FBS, 100 mM dexamethasone, 0.2 mM L-ascorbate-2-phosphate and 10 mM β-glycerophosphate (Sigma-Aldrich). The medium was changed every other day for 21 days. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and stained with Alizarin Red (Sigma-Aldrich) to detect the bone matrix mineralization. To induce adipogenic differentiation, cells were induced by 10 μg/ml insulin, 60 μM indomethacin, 0.5 μM hydrocortisone and 0.5 mM isobutyl methylxanthine (IBMX) for 21 days. Cells were then fixed and lipid droplets were stained by Oil Red O (Sigma-Aldrich). For chondrogenic differentiation, hPMSCs were induced by ITS-plus premix (BD Biosciences, San Jose, CA) at a concentration of 6.25 μg/ml insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenium acid. Additionally, 50 μg/ml ascorbate 2-phosphate, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate, 100 mM dexamethasone and 10 ng/ml TGF-β3 were also added. After 21 days, chondrocytes were stained with Alcian Blue (Sigma-Aldrich).

Flow cytometry analysis. hPMSCs were collected and washed twice in phosphate-buffered saline (PBS) and resuspended at a concentration of 1x10⁶ cells/ml. 10 μl of the detecting antibody was added to 100 μl of the cell suspension. Fluorescence-conjugated antibodies against CD73, CD105, CD90, CD34 and CD45 (Biolegend, San Diego, CA, USA) were incubated with cells at 37°C for 30 min. Cells were then analyzed using a FACS Calibur instrument and CellQuest Pro software version 3.3 (BD Biosciences, San Jose, CA, USA).

Differentiation of hPMSCs into neural-like cells. hPMSCs were seeded at a density of 2x10⁴ cells/well onto a 6-well plate (Nunc), coated with Geltrex (Gibco). Neural differentiation was induced by the induction medium, containing Neurobasal (NB) medium (Gibco), DMEM/F12 (Hyclone), N2 supplement (100X, Gibco), and 10 μM of retinoic acid (Gibco). In addition, neural differentiation was performed by a 3-step protocol. Firstly, the induction medium was supplemented with DAPT at various concentrations (0, 5, 10 and 20 μM) for 7 days. Then, 10 μM 5-Azacytidine (Sigma-Aldrich) was added in the induction medium with 10 μM DAPT for another 7 days. Finally, either 10 μM VPA or 10 μM chloroquine was supplemented into the differentiation medium, that already included 10 μM of DAPT and 10 μM of 5-Azacytidine. Differentiated cells were collected at day 1, 5 and 7 for RT-PCR gene expression analysis and cell morphology was observed by a phase-contrast microscope.
Immunocytochemistry. Cells were washed with 3X PBS, and fixed with 4% PFA for 15 min. Then, the cells were incubated in blocking buffer, which included 4% bovine serum albumin (BSA; Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 1 h. Primary antibodies were diluted in a blocking solution at the followed dilutions; anti-βIII-tubulin (1:1,000; Sigma-Aldrich), anti-NESTIN (1:500; MERK), anti-LC3I/II (1:1,000; MERK), anti-CD90-FITC (1:500; MERK), anti-Endoglin-CD105 (1:200; MERK) and anti-CD73 (1:500; MERK). Primary antibodies were applied overnight at 4˚C. Cells were then washed 3X with washing buffer (PBS, 0.1% Triton X100), followed by the incubation of secondary antibodies conjugated with either Alexa fluorophore 488 or 593 at 1:1,000 for 1 h at room temperature. Cells were washed 3 times in PBS and incubated with DAPI for nuclear staining (Biorad; Hercules, CA, USA).

Reverse transcription polymerase chain reaction (RT-PCR). Cells were detached by 0.25% trypsin-EDTA (Hyclone) and centrifuged at 11,000 rpm for 5 min. Total RNA was extracted by the RNA minikit (Nucleospin; Duran, Germany). 300 ng of total RNA sample were used for each reverse transcription with the cDNA kit (Toyobo; Osaka, Japan). Primers of PCR were obtained from Macrogen (Seoul, Republic of Korea) and are listed in Table I. GAPDH was used as an internal control gene to normalize gene expression in each sample. cDNA was amplified by a thermal cycler PCR machine (Bio-Rad).

Statistical analysis. Results from different experiments were analyzed using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as means±standard deviation (SD). Statistical analysis of the data was performed using one-way ANOVA, followed by the Turkey post-hoc test. A p-Value <0.05 denoted the presence of a statistically significant result, whereas p<0.01 was considered highly significant.

Results

Derivation and Characterization of hPMSCs from human placental tissues. We isolated hPMSCs from human placental tissues of by the serial digestion of collagenase/displace enzyme. hPMSCs adhered onto a culture dish after a 24-h isolation and cell migration was detected after 3 days. Interestingly, hPMSCs showed a fibroblast-like structure, and reached 100% confluency at day 14 post-isolation. hPMSCs were then harvested by 0.25% trypsin and expanded to passage 1 (Figure 1A). The expression of several cell surface protein markers, typical of hPMSCs and MSCs and indicative of the differentiation state, was analyzed using immunofluorescence and flow cytometry. Immunostaining results showed that hPMSCs strongly expressed CD73, CD90 and CD105 (Figure 1B). Identification of MSC characteristics by flow cytometry emphasized the homogeneity of hPMSCs, regarding MSC cell surface markers. The major population of hPMSCs were positively marked by CD73, CD90 and CD105, but not by hematopoietic stem cells markers CD34 and CD45 (Figure 1C). Finally, confirming their mesenchymal characteristics, we found that hPMSCs had an osteogenic, chondrogenic and adipogenic potential. The multipotent differentiation of hPMSCs was then examined by inducing mesodermal differentiation into osteogenic, chondrogenic, and adipogenic lineages. After 21 days of induction, hPMSCs possessed the typical mesenchymal stem cell differentiation propensity and became osteocytes, chondrocytes and adipocytes. The resulting adipocytes exhibited lipid droplet-containing cells as stained by Oil Red O, while chondrocytes and osteocytes were detected by Alcian Blue and Alizarin Red, respectively (Figure 1D).

Inhibition of notch signaling induced neural differentiation and upregulated autophagy genes in hPMSCs. The involvement of Notch signaling in neural differentiation has previously been investigated (25, 26). We aimed to explore the effect of inhibiting Notch signaling and whether it can influence neural differentiation of hPMSCs. DAPT, a γ-
secretase inhibitor, was used for inhibiting Notch signaling and induce neural differentiation of hPMSCs. Various concentrations of DAPT (0, 5, 10 and 20 μM) were supplied into the differentiation culture for 7 days prior to assessing the expression of neural genes. At day 7, the structure of hPMSCs in 5, 10, 20 μM DAPT presented a spindle-shaped morphology (Figure 2A). Neuronal genes, including Nestin, βIII-tubulin, and SOX2, were significantly upregulated when 10 and 20 μM of DAPT was applied (Figure 2B, C). Interestingly, the autophagy genes LC3I/II and Beclin were also significantly upregulated when Notch signaling was blocked by 10 and 20 μM DAPT, compared to the undifferentiated control cells (Figure 2D, E), suggesting that autophagy plays a role in the differentiation process. These data exhibited that DAPT promoted neural differentiation and enhanced the activity of autophagy in hPMSCs.

Figure 1. Isolation and characterization of human placenta-derived stem cells. (A) Bright field images of cellular morphology of primary culture expanded from placental tissues. Fibroblast-like cells of hPMSCs emigrated from digested placental tissues and adhered to the culture dish at day 3, 5 and 14 post-isolation, and hPMSCs at passage 1. (B) Immunocytochemistry of hPMSCs for mesenchymal stem cell surface antigens including CD73, CD90 and CD105. (C) A major population of hPMSCs was positive for CD73, CD90 and CD105, and negative for CD34 and CD45, as analyzed by flow cytometry. (D) Multipotent differentiation of hPMSCs for osteocytes, chondrocytes, and adipocytes was demonstrated by lipid droplets with Oil-Red O, mineral nodules with Alizarin red and proteoglycan matrix of Alcian blue.
5-Azacytidine enhanced neural differentiation efficiency and activated autophagy of hPMSCs. More recent studies have examined how autophagy could enhance neural differentiation; recent evidence show autophagy is involved in the efficiency of stem cell differentiation. In addition to DNA demethylating agents, a number of studies have found 5-Azacytidine can induce autophagy in various contexts (27, 28). In this regard, the ability of 5-Azacytidine in enhancing neural differentiation was evaluated. hPMSCs were differentiated into neural-like cells in the differentiation media with either 10 μM DAPT or 10 μM 5-Azacytidine alone or in combination. After 7 days of differentiation, the morphology of hPMSCs became neural in all differentiation conditions, compared with the undifferentiated control cells (Figure 3A). The combined treatment of DAPT and 5-Azacytidine resulted in the highest expression of neural genes, Nestin and βIII-tubulin, as well as GAD1, a marker of glutaminergic neurons as measured by RT-PCR (Figure 3B, C). Immunofluorescence confirmed that the combined treatment of DAPT and 5-Azacytidine induced the neural markers Nestin and βIII-tubulin in differentiated hPMSCs (Figure 3D). This result indicates that 5-Azacytidine could enhance the efficiency of neural differentiation of hPMSCs. Furthermore, the influence of 5-Azacytidine in autophagy was investigated. The expression of LC3I/II and BECLIN was determined after 7 days of neural differentiation in various conditions; either 10 μM DAPT or 10 μM 5-Azacytidine alone or in combination. The combined treatment of DAPT and 5-Azacytidine significantly augmented the expression of LC3I/II and BECLIN genes,
compared to the undifferentiated control and DAPT treatment alone (Figure 4A, B). LC3I/II and BECLIN were also found to co-localize with βIII-tubulin in hPMSCs-derived neural cells, validating the activation of autophagy (Figure 4C). This data suggested that supplying 5-Azacytidine in inductive medium could promote autophagic activity resulting in enhanced neural differentiation.

**Modulation of autophagy influenced the efficacy of neural differentiation of hPMSCs.** We next confirmed the influence of autophagy during neural differentiation of hPMSCs. To address this, we applied small molecules are known to modulate autophagy. Chloroquine (CQ) or Valproic acid (VPA) were used to either block or enhance autophagy within cells. The changes of cellular morphology were
observed at day 1, 5 and 7 of differentiation. Compared to the differentiation control, VPA further induced spindle-like cells, while CQ caused prominent cell death (Figure 5A). The expression of βIII-tubulin and LC3I/II was assessed at day 1, 5, and 7 of differentiation by RT-PCR (Figure 5B). Compared to control, VPA significantly enhanced LC3I/II expression at day 7, while CQ did not have a notable change (Figure 5C). βIII-tubulin was also significantly increased in VPA-induced hPMSCs and its level was clearly suppressed in CQ-supplemented conditions (Figure 5C). Consistently, immunofluorescence results showed that the treatment of chloroquine led to the reduction of βIII-tubulin-positive cells, in contrast to VPA-treated hPMSCs (Figure 5D). These results propose that neural differentiation of hPMSCs can be modulated by autophagy within cells; the more autophagic activity, the higher the neural differentiation efficiency.
Figure 5. The modulation of autophagy altered neural differentiation of hPMSCs. (A) Morphological changes of hPMSCs during neural differentiation, when either an autophagy activator (Valproic acid; VPA) or an autophagy inhibitor (chloroquine; CQ) was supplemented. (B) The expression of βIII-tubulin and LC3I/II was determined by RT-PCR. (C) VPA significantly enhanced the expression of LC3I/II and βIII-tubulin, compared with the differentiated control cells. Data are shown as mean±SD. *p<0.05 vs. the differentiated control cells. (D) The immunofluorescent images showed colocalization of βIII-tubulin and LC3I/II when VPA was added to the cells.
Discussion

In this study, we demonstrated that hPMSCs isolated from the placental tissues exhibited fibroblastic appearance in primary culture and maintained stemness by expression of MSCs markers (CD73, 90, 105), but not hematopoietic stem cell (HSC) markers (CD34, 45). However, it has been suggested that when the isolated hPMSCs are MSC-like, they present immunoprivileged properties which would not cause an inflammatory response (29). Our first goal was to determine whether the isolated hPMSCs maintained their multiple lineage differentiation ability. Similar to others, we found that hPMSCs in this study possessed the differentiation capacity into adipocytes, osteocytes, and chondrocytes (30-32).

This study set out with the aim of assessing the differentiation efficiency, and we were able to induce hPMSCs towards neurons by inhibiting Notch signaling and DNA methylation. Notch signaling plays a critical role in development and cell fate specification. Notch receptors and ligands have been found to be expressed and play fundamental roles during the developmental process of the placenta (33). Prior studies have suggested that inhibition of Notch signaling is associated with neuronal differentiation (26, 34). Notch signaling can be suppressed by a γ-secretase inhibitor, DAPT (35). Thus, we used DAPT in varying concentrations together with N2 medium and retinoic acid, which have the ability to induce post-mitotic, neural phenotypes in various types of stem cells (36, 37). Upon induction, we could detect increasing expression of βIII-tubulin Nestin, and SOX2 in cells treated with DAPT during the neural differentiation protocol. Only cells treated with 10 and 20 μM were found to clearly express SOX2, therefore, we chose DAPT at a concentration of 10 μM for Notch inhibition and further experiments. We observed increased expression of autophagy genes (LC3I/II, BECLIN) upon treatment with DAPT in a dose-dependent manner. Hence, it could be hypothesized that autophagy plays a role in neuronal differentiation once Notch singling is inhibited.

We further examined the potential of DAPT and 5-Azacytidine in enhancing neuronal differentiation. Notably, the neural marker genes were utmost upregulated when combining DAPT and 5-Azacytidine. High expression of both Nestin and βIII-tubulin in hPMSCs was observed. This indicated that hPMSCs can efficiently differentiate into neural-like cells with DAPT treatment, and 5-Azacytidine could further enhance neuronal differentiation. hPMSCs are able to differentiate into neural precursors, but limited evidence was found in prior studies on hPMSCs differentiation into mature neurons (38, 39). Intriguingly, we further found GAD1, a marker of glutaminergic neurons, was highly expressed when hPMSCs were simultaneously treated with DAPT and 5-Azacytidine. The neural markers we characterized included both Nestin and βIII-tubulin. Nestin is known to be expressed within mesenchymal stem cells, and the expression of Nestin was increased when cells differentiated into neurons (40). βIII-tubulin is expressed after neuronal differentiation and utilized as a marker of mature neurons during the final stages of growth (41, 42). Our results were similar to a previous study that reported Notch inhibition by DAPT and subsequent differentiation of human induced pluripotent stem cells towards a neural fate (43).

In addition, the epigenetic modifier 5-Azacytidine was used to enhance the neural differentiation potential in this study. In 2013, Zemelko and colleagues reported that retinoic acid (RA) in combination with 5-azacytidine caused the
elevation neurogenic potential of adipose-derived MSCs (44). Another study reported that adipose-derived mesenchymal stem cells (ASCs) maintained in the medium with RA and 5-Azacytidine for 7 days had increased mRNA and protein levels of Nestin and βIII-tubulin (45). Similar to our study, the combination 5-Azacytidine with DAPT medium promoted a neural-like cell production from hPMScs-MSCs at a greater rate than DAPT alone. Thus, it could be suggested that only two small molecules, 5-Azacytidine cytidine and DAPT, can potentially direct hPMSC reprogramming toward neuronal cells. In addition to the inhibition of Notch signaling, expression of autophagy genes (LC3I/II, BECLIN) was enhanced upon DAPT treatment, in a dose-dependent manner. Most recent data show that bone marrow mononucleated cells exposed to 5-Azacytidine express increased proteins associated with autophagy (46). Hence, it could be hypothesized that autophagy plays a role in neuronal differentiation, once Notch singling is inhibited. In line with the previous observation, the expression of LC3I/II, an autophagy marker, peaked after the induction and demonstrated the same trend as neural differentiation (47). Moreover, autophagic activity was upregulated during the neuronal differentiation of Neuro 2A (N2a) cells (48). In previous studies, autophagy was shown to play an important role in support and protection of cells during differentiation (49-53). This evidence implies that autophagic activity may be an important supportive condition in neural cell differentiation, and it is activated in response to the neural differentiation signals (Figure 6).

Therefore, it is promising that there are connections between autophagy and the neural differentiation of hPMSCs. We validated the role of autophagy in this regard by using chemical modulators; an autophagy activator and an autophagy inhibitor. Valproic acid (VPA), a histone deacetylase inhibitor (HDAC), has been widely used for activating autophagy, which could then induce cell differentiation (54). Chloroquine is an anti-malarial drug, and it is generally accepted as an autophagy inhibitor that leads to a massive accumulation of ubiquitinated LC3I/II (55). Besides autophagy activation, the treatment of VPA promotes neural markers, contrasting with the addition of chloroquine and the control. These data propose that neural differentiation of hPMSCs occurs in conjunction with the activation of autophagy (56, 57). However, there are more complex signaling factors involved in the regulation of neuronal differentiation and the mechanistic relationship between Notch signaling and autophagy requires further exploration.

Conclusion

The combination of DAPT and 5-Azacytidine efficiently induced neural differentiation of hPMSCs and autophagy was significantly activated upon stem cell differentiation. The levels of autophagic activity positively correlated with the efficiency of neural differentiation, suggesting that autophagy might play a role in regulating stem cell differentiation (Figure 6).

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors’ Contributions

Areechun Sothibundhu provided concept and design of the study, performed experiments, analyzed and interpreted data and prepared the article for publication. Pattamon Muangchan performed experiments, analyzed and interpreted data. Ruchee Phonchai performed experiments, provided resources, prepared the article for publication. Wilasinee Promjantuek collected samples, analyzed and interpreted data. Niph Chaicharonaudomrung analyzed and interpreted data and prepared the article for publication. Phongsakorn Kunhorm analyzed and interpreted data and prepared the article for publication. Parinya Noisa provided the concept and design of the study, collected samples, analyzed and interpreted data, wrote the manuscript, prepared the article for publication and gave the final publication approval of the manuscript.

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