GAPDH is critical for superior efficacy of female bone marrow-derived mesenchymal stem cells on pulmonary hypertension

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Aims

Pulmonary arterial hypertension, a chronic lung disease, remains an unacceptable prognosis despite significant advances in conventional therapies. Stem cell therapy represents a novel and effective modality. This study was aimed to add new insight in gender differences of bone marrow-derived mesenchymal stem cells on therapy against pulmonary arterial hypertension and the underlying mechanism.

Methods and results

By \textit{in vivo} experiments, we showed for the first time female bone marrow-derived mesenchymal stem cells possessed a better therapeutic potential against monocrotaline-induced pulmonary arterial hypertension in C57BL/6\textit{j} mice compared with male counterparts. \textit{In vitro} experiments demonstrated superior function of female bone marrow-derived mesenchymal stem cells in cell proliferation, migration and $[\text{Ca}^{2+}]_i$ kinetics. Moreover, we unexpectedly found that, compared with male ones, female bone marrow-derived mesenchymal stem cells had a higher expression level of glyceraldehyde-3-phosphate dehydrogenase and manipulations of its expression in female or male bone marrow-derived mesenchymal stem cells profoundly affected their cellular behaviours and therapeutic efficacies against pulmonary arterial hypertension.

Conclusion

Our results suggest that glyceraldehyde-3-phosphate dehydrogenase plays a critical role in determining the superior functions of female bone marrow-derived mesenchymal stem cells in cell therapy against pulmonary arterial hypertension by regulating $[\text{Ca}^{2+}]_i$ signal-associated cellular behaviours.

Keywords

Pulmonary arterial hypertension ● Glyceraldehyde-3-phosphate dehydrogenase ● Bone marrow-derived mesenchymal stem cells ● Gender

1. Introduction

Pulmonary arterial hypertension (PAH), a disorder that is closely associated with chronic inflammation and can be rapidly progressive and fatal, is characterized by elevated pulmonary artery pressure, arteriolar wall remodelling and right ventricular hypertrophy (RVH).\textsuperscript{1,2} Despite significant advances in conventional therapies for PAH during the past decades, the prognosis remains very poor.

Recent investigations have suggested that mesenchymal stem cells (MSCs) therapy represents a novel and effective modality.\textsuperscript{3,4} Interestingly, the potential importance of gender difference of stem cells on therapeutic efficiency were emphasized in animal studies on several types of diseases including fat tissue engineering,\textsuperscript{5} ischaemia/reperfusion injury,\textsuperscript{6} and articular cartilage defect model.\textsuperscript{6} Ogawa et al.\textsuperscript{6} showed that adipose-derived stromal cells (ASCs) harvested from female mice differentiated more efficiently into adipocytes than those from male mice, indicating a greater value of female ASCs on fat augmentation surgery such as breast reconstruction. In a study on ischaemia/reperfusion injury, female MSC-treated rat hearts demonstrated significantly a greater recovery of left ventricular function than male MSC-treated ones at the end of reperfusion.\textsuperscript{5} Contrarily, there was also an important study showing better therapeutic potential of male stem cells than female
counterparts in an articular cartilage defect model. The discrepancy suggests a possibility that the stem cell sex difference on therapeutic efficiency can be disease or tissue specific. Additionally, the underlying mechanism for gender differences of MSCs is likely multi-factorial. Some studies implied a major role of sex steroid hormones. Others indicated that female MSCs produced more growth factor and less inflammatory tumour necrosis factor alpha under stress. However, the mechanism underlying the distinct behaviour of female and male stem cells, particularly the intrinsic difference between female and male stem cells, remains largely unknown. Furthermore, to the best of our knowledge, there is no any report about gender difference of MSCs on therapy against PAH.

2. Methods

For details regarding methods, refer to the Supplementary material online, Methods.

2.1 Ethics statement and the animals used

C57BL/6j mice were used for stem cell isolation and transplantation as well as PAH models. All animal experiments were approved by the Institutional Animal Care and Use Committee.

2.2 Primary culture of MSCs

Bone marrow-derived mesenchymal stem cells (BMSCs) were obtained by flushing the femur and tibia diaphysis, cultured, and identified using Fluorescence Activated Cell Sorting (FACS).

2.3 In vivo experimental protocol

Female and male C57BL/6j mice, 20–25 g, 8–10 week old, received monocrotaline (MCT, 60 mg/kg) via tail vein to produce the PAH model. BMSCs therapy protocol and experimental groups are available in Supplementary material online. Thirty days later, Fulton’s index, the ratio of right ventricle weight over the weight of the left ventricle plus the septum RV/(LV+S) was measured and lungs were harvested for the assessment of pulmonary artery muscularization.

2.4 Measurement of [Ca\(^{2+}\)]\(_i\) concentration in response to histamine and H\(_2\)O\(_2\)

[Ca\(^{2+}\)]\(_i\) was continuously monitored before and after the stimulations of 10 μM histamine or 100 μM H\(_2\)O\(_2\) in BMSCs as we previously described in detail.

2.5 Cell proliferation and migration assay

Cell proliferation was measured by an MTS colorimetric assay according to manufacturer’s instructions (Cell Titer, Promega). BMSC migration assays were performed using 8-mm pore size chemotaxis filters within Boyden chamber inserts (Costar).

2.6 Western blot assay

GAPDH levels were analysed by western blot using specific antibody (Cell Signaling Technologies) according to the manufacturer’s instructions. β-Actin and α-tubulin together served as a control for equal protein loading.

2.7 Plasmids and stable transfection

BMSCs in exponential growth were concurrently transfected with 2 μg of a siRNA vector (pGCsilenicerTM U6/Neo/green fluorescent protein (GFP)/RNAi vector, Genechem) or GAPDH-specific siRNA (pGCsilenicerTM U6/Neo/GFP/RNAi-GAPDH, Genechem) for female groups, or with an over-expression vector (pIREs2-ZsGreen1 vector, BioWit Technologies) or GAPDH-over-expressing plasmid (pIREs2-ZsGreen1-GAPDH, BioWit Technologies) for male groups.

2.8 MSC differentiation and immunohistochemistry in vivo

To detect localization and differentiation of transplanted GFP-expressing BMSCs into endothelial cells, immunohistochemistry for the lung tissues from recipients was performed using antibodies against GFP (Invitrogen) and von Willebrand Factor (vWF) (Santa Cruz Biotecnology).

2.9 Determination of NAD\(^+\)/NADH

NAD\(^+\)/NADH in BMSCs was determined using the Amplitite Colorimetric NAD\(^+\)/NADH Assay Kit (AAT Bioquest) according to manufacturer’s protocols.

2.10 Statistical analysis

Data are reported as means ± SD of at least three independent experiments. SNK-q test and one-way analysis of variance analysis were used for two or multiple group comparisons. P < 0.05 was accepted as indicating statistical significance.

3. Results

3.1 Characterization of BMSCs

Mononuclear cells from male and female C57BL/6j mice of the same litter bone marrow were successfully isolated, expanded, and characterized. The cells were homogeneously long rhombus, fibroblast-like (Supplementary material online, Figure S1A). We further identified BMSCs by FACS analysis. Over 99% cells expressed a typical MSC marker CD29, while rarely expressed haematopoietic markers CD45 and CD34 (0.34 ± 0.13%) (Supplementary material online, Figure S1B–F).

3.2 Gender differences of BMSCs on therapeutic efficiency for MCT-induced PAH

Both the female and male mice treated with 3 weeks of MCT injection fully developed PAH9,10 as illustrated by its resultant changes including elevations in RV/(LV+S) (P < 0.05, Figure 1A and B) and muscularization of pulmonary vessels (P < 0.05, Figure 1C–F). Then PAH mice were untreated, treated with female, or male BMSCs. Regardless of gender of the receiver, female and male BMSCs both significantly decreased Fulton’s index RV/(LV+S) (Figure 1A and B), an index of RVH.17 Moreover, female BMSCs conferred markedly greater improvement of RV/(LV+S) (P < 0.05). We also measured degree of muscularization in pulmonary resistance vessels (25–100 μm in diameter) to evaluate pulmonary vascular remodelling. Similarly, though male BMSCs treatment decreased degree of muscularization in both genders of MCT-injected mice (P < 0.05), female BMSCs always showed a significantly better therapeutic efficacy (P < 0.05, Figure 1C–F). Taken together, the above results indicated that both male and female BMSCs were effective against MCT-induced PAH in both genders of the mice, and female BMSCs possessed a better therapeutic efficiency than male cells either in a sex-matched or in a mismatched way.

3.3 Gender differences in [Ca\(^{2+}\)]\(_i\), signal kinetics and cellular behaviours

Previous studies including ours showed that stimulation of inflammatory mediators mimicking the pathophysiologically relevant circumstances produced cellular downstream events through [Ca\(^{2+}\)]\(_i\), signal kinetics.13 We sought to investigate whether [Ca\(^{2+}\)]\(_i\), signalling was involved in the gender difference of BMSCs in response to histamine and H\(_2\)O\(_2\), the
results were obtained from H2O2-stimulated BMSCs. Eighty-one female and three male BMSCs showed no response to histamine stimulation. Similar to above, a few female cells (8/53, 15%) showed sustained \([\text{Ca}^{2+}]_{i}\) elevation (7/37, 19%), and the majority of male BMSCs (23/31, 74%) exhibited sustained \([\text{Ca}^{2+}]_{i}\) elevation (27/34, 79.5%) and a few female cells (8/53, 15%) showed sustained \([\text{Ca}^{2+}]_{i}\) elevation (7/34, 21%). In contrast, a few female cells (6/34, 17.5%) and 1 cell showed no response. Data were obtained from at least three separate experiments for each. (E) The growth curve of the cultured BMSCs at passage 3. Each point represented the mean value of triplicate measurements. (F) The proliferation assay of BMSCs with or without histamine or \(\text{H}_2\text{O}_2\) stimulation. On Day 1, the female and male BMSCs were treated with 10 \(\mu\text{M}\) histamine or 100 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) or neither of them, the female and male BMSCs were allowed to migrate for 3 h separately before being fixed and stained, and the number of migrated BMSCs on the lower side of the filters were counted by microscopy. Both female and male BMSCs migration in response to histamine and \(\text{H}_2\text{O}_2\) stimulation were evaluated as the fold increase vs. blank control of F-BMSCs. Results represented the mean of three independent experiments, each performed in triplicate (\(*P < 0.05\)).
stimulation generated \([\text{Ca}^{2+}]\), oscillations in most of female BMSCs and sustained \([\text{Ca}^{2+}]\), elevation in most of male BMSCs, indicating the presence of significant gender difference of BMSCs in \([\text{Ca}^{2+}]\), signal kinetics under the pathological circumstance of PAH.

Increasing evidence indicates that \([\text{Ca}^{2+}]\) regulates diverse cellular behaviours in response to various stimuli through its kinetics. \([\text{Ca}^{2+}]\) oscillations can effectively stimulate gene expression,\textsuperscript{11,18} while sustained increase of \([\text{Ca}^{2+}]\) may induce cell damage.\textsuperscript{19,20} Therefore, we further examined whether histamine or \(\text{H}_2\text{O}_2\) stimulation, which triggered the different \([\text{Ca}^{2+}]\), kinetics in female and male BMSCs, produced any gender-specific difference in cellular behaviours.

Under current experimental conditions, the BSMCs grew in a triphasic way: the lag phase from Day 0 to Day 2, the exponential phase from Day 2 to Day 7, and the plateau phase after the 7th day (Figure 2E), and female BMSCs grew at a higher speed than male counterparts after the lag phase. To reveal if there was sex difference in BMSCs proliferation under the conditions simulating PAH circumstance, the female and male BMSCs grew on Day 1, the lag phase, were treated with histamine or \(\text{H}_2\text{O}_2\). Both histamine and \(\text{H}_2\text{O}_2\) stimulation elicited a rapid growth in female BMSCs, not in male BMSCs (Figure 2F). The cell migration assay showed that female BMSCs had a much higher basal migration capacity \((P < 0.05)\). More importantly, histamine and \(\text{H}_2\text{O}_2\) treatment significantly enhanced the migration of female, not male BMSCs \((P < 0.05, \text{Figure} \ 2G)\).

Collectively, the priority of female BMSCs in \([\text{Ca}^{2+}]\), kinetics and cellular behaviours may depend on an intrinsic mechanism(s) between female and male BMSCs.

**Figure 3** Manipulation of GAPDH protein expression reversed gender differences of BMSCs in \([\text{Ca}^{2+}]\), kinetics. (A) Representative western blot assay for GAPDH in BMSCs, \(\beta\)-actin, and \(\alpha\)-tubulin together served as a control for equal protein loading. (B) Representative western blot assay for GAPDH in female and male BMSCs (F-BMSCs and M-BMSCs) after transfection with GAPDH-over-expressing (OE-GAPDH) or siRNA plasmids (siRNA-GAPDH), \(\beta\)-actin, and \(\alpha\)-tubulin together served as a control for equal protein loading. (C and D) Band densities of GAPDH. The GAPDH density was normalized by \(\beta\)-actin and \(\alpha\)-tubulin level and then the mean value of each group expressed as a percentage of the F-BMSCs control group. \(n = \ 4\) for each. *\(P < 0.05\). (E) Representative \([\text{Ca}^{2+}]\), oscillations from a siRNA vector-transfected-F-BMSCs monolayer exposed to 10 \(\mu\)M histamine in HBS. (F) Representative \([\text{Ca}^{2+}]\), elevation from a siRNA-GAPDH-transfected-F-BMSCs monolayer exposed to 10 \(\mu\)M histamine in HBS. (G) Representative \([\text{Ca}^{2+}]\), elevation from a OE vector-transfected-M-BMSCs monolayer exposed to 10 \(\mu\)M histamine in HBS. (H) Representative \([\text{Ca}^{2+}]\), oscillations from a OE-GAPDH-transfected-M-BMSCs monolayer exposed to 10 \(\mu\)M histamine in HBS.
3.4 GAPDH is critical in gender differences of BMSCs on \([\text{Ca}^{2+}]\), kinetics and cellular behaviours

By accident, we found a higher expression level of GAPDH protein in female vs. male BMSCs \((P < 0.05, \text{Figure } 3A \text{ and } C \text{ and Supplementary material online, Figure S3})\). Noteworthy, both histamine and \(\text{H}_2\text{O}_2\) stimulation distinctly increased GAPDH expression in female BMSCs \((P < 0.05)\), not in male BMSCs (Figure 3A and C). These results indicated that histamine and \(\text{H}_2\text{O}_2\) treatment magnified the innate difference of the GAPDH protein level between female and male BMSCs.

To elucidate whether GAPDH is responsible for gender differences of BMSCs on \([\text{Ca}^{2+}]\), kinetics, we constructed GAPDH-over-expressing plasmid (OE-GAPDH) as well as specific siRNA against GAPDH and confirmed their efficiency by the western blot assay (Figure 3B and D). Then we decreased the GAPDH protein level by siRNA-mediated knockdown in female BMSCs and the \([\text{Ca}^{2+}]\), kinetics after histamine stimulation were examined. When compared with the control group in which the female cells were transfected with vehicle siRNA, 48 out of a total 70 cells (48/70, 65%) generated \([\text{Ca}^{2+}]_i\), oscillations, in the GAPDH-specific siRNA-treated group the majority cells (38/67, 57%) manifested sustained \([\text{Ca}^{2+}]_i\), elevations, instead, only 24% cells (16/67) generated \([\text{Ca}^{2+}]_i\), oscillations (Figure 3E–F, Supplementary material online, Figure S2E). The experiments in male BMSCs using OE-GAPDH further confirmed that the GAPDH protein level determines the different \([\text{Ca}^{2+}]_i\), kinetics. In the vehicle control group, sustained \([\text{Ca}^{2+}]_i\), elevations were found in most male cells after 10 \(\mu\text{M}\) histamine stimulation (52/76, 68%), whereas in the OE-GAPDH group, 36 out of a total 56 transfected male BMSCs (64%) treated with histamine showed \([\text{Ca}^{2+}]_i\), oscillations (Figure 3G–H and Supplementary material online, Figure S2E). Similarly, \(\text{H}_2\text{O}_2\)-stimulated \([\text{Ca}^{2+}]_i\), signalling was altered both in male and female BMSCs with manipulated the GAPDH expressing level (Supplementary material online, Figure S2A–D and F). These data suggested that manipulation of GAPDH protein expression was able to reverse gender difference-associated \([\text{Ca}^{2+}]_i\), kinetics under histamine and \(\text{H}_2\text{O}_2\) stimulation.

As shown in Figure 4A, the growth curves of female and male BMSCs apparently changed after GAPDH knockdown or over-expression, respectively. The growth speed comparison indicated that up-regulation of GAPDH protein promoted proliferation in male BMSCs, whereas down-regulation of GAPDH inhibited female BMSCs proliferation. By histamine and \(\text{H}_2\text{O}_2\) treatment, siRNA knockdown of GAPDH in female BMSCs abolished its priority on cell proliferation and migration in response to the stimuli. Meanwhile, over-expression of GAPDH protein in male BMSCs promoted their proliferation and migration property after histamine and \(\text{H}_2\text{O}_2\) stimulation (Figure 4B–E). Collectively, gender differences of BMSCs on \([\text{Ca}^{2+}]_i\), kinetics and cellular behaviours can be reversed by manipulating GAPDH expression.

3.5 Manipulation of GAPDH protein expression reversed the gender differences of BMSCs on their therapeutic efficiencies against PAH

Previous studies including ours indicate that \([\text{Ca}^{2+}]_i\), signalling plays a central role in the development and the progression of PAH due to its involvement in both vasoconstriction and vascular remodelling.\(^{14,21}\)

On the basis of above in vitro findings in the current study, we examined whether the gender differences between female and male BMSCs on therapeutic efficiency against MCT-induced PAH was affected by the GAPDH protein expression level. As shown in Figure 5A–D, siRNA knockdown of GAPDH resulted in the functional loss of female BMSCs on their superior improvement of RV/(LV+S) and pulmonary arterial muscularization \((P < 0.05)\), whereas male BMSCs over-expressing GAPDH gained greater therapeutic efficiency against PAH by decreasing RV/(LV+S) and degree of arterial muscularization \((P < 0.05)\). Furthermore, by immunohistochemistry, exogenous GFP-expressing BMSCs were found to have migrated to pulmonary blood vessels and differentiated into endothelial cells (Figure 5E), with these abilities greatly influenced by GAPDH (Figure 5F and G).

3.6 Possible mechanism for GAPDH regulating \([\text{Ca}^{2+}]_i\), kinetics

Our data suggested a strong link between GAPDH and \([\text{Ca}^{2+}]_i\), kinetics as well as its following downstream cellular events. We here performed experiments to investigate the possible mechanism for GAPDH regulation on \([\text{Ca}^{2+}]_i\), kinetics. On the basis of recent findings around close relationship among GAPDH, inositol 1,4,5-trisphosphate receptors (IP\(_3\)R), NADH, and \([\text{Ca}^{2+}]_i\), signalling,\(^{22,23}\) we detected NAD\(^+\)/NADH in both gender of BMSCs, treated or untreated with dicumarol (DIC)
or β-Lapachone (BL) to inhibit or activate NAD(P)H:quinone oxidoreductase (NQO1).\textsuperscript{24,25} The results indicated that the NAD\textsuperscript{+}/NADH ratio was higher in female BMSCs than in male cells (Figure 6A), consistent with the GAPDH expression level as showed in above data. Reversing NAD\textsuperscript{+}/NADH ratio in female or male BMSCs by DIC or BL, [Ca\textsuperscript{2+}]i signalling, cell proliferation, and migration after histamine or H\textsubscript{2}O\textsubscript{2} became at least partially reversed (Figure 6B).

### 4. Discussion

Gender differences on stem cell therapy have been documented recently. Consistent with the previous studies on fat tissue augmentation and ischaemia/reperfusion injury,\textsuperscript{4,5} female BMSCs conferred greater protection against MCT-induced PAH than male BMSCs. Oppositely, in an articular cartilage defect rat model,\textsuperscript{6} male BMSCs showed a better...
therapeutic potential than female counterparts. These findings emphasized the importance about gender differences of stem cells and the source gender a significant determinant for therapeutic efficiency. They also indicate that gender differences of stem cells are tissue or disease specific. Therefore, the examination of gender difference of stem cell therapy for different diseases like PAH in the current study becomes necessary and the understanding of underlying mechanisms would enable us to have better therapeutic strategies.

Several recent reports have indicated that sex hormones influence the cellular activities of stem cells. In the current study, the comparison of sex-match and mismatch transplantations revealed that the sex of host tissue did not affect therapeutic efficiency of either female or male.

Figure 6 Possible role of \( \text{NAD}^{+}/\text{NADH} \) in \( \text{GAPDH} \) regulated \( [\text{Ca}^{2+}] \) kinetics and cellular behaviours. (A) \( \text{NAD}^{+}/\text{NADH} \) ratios in F-BMSCs and M-BMSCs with or without DIC or \( \beta \text{L} \) (compared with the F-BMSCs group). (B) Representative \( [\text{Ca}^{2+}] \) elevation from an F-BMSCs monolayer exposed to 100 \( \mu \text{M} \) DIC and 10 \( \mu \text{M} \) histamine in HBSS. (C) Representative \( [\text{Ca}^{2+}] \), elevation from an F-BMSCs monolayer exposed to 100 \( \mu \text{M} \) DIC and 100 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_2 \). (D) Representative \( [\text{Ca}^{2+}] \), oscillations from a M-BMSCs monolayer exposed to 2 \( \mu \text{M} \) \( \beta \text{L} \) and 10 \( \mu \text{M} \) histamine. (E) Representative \( [\text{Ca}^{2+}] \), elevation from a M-BMSCs monolayer exposed to 2 \( \mu \text{M} \) \( \beta \text{L} \) and 100 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_2 \). (F and G) Summarization of \( [\text{Ca}^{2+}] \) response in all tested female and male BMSCs. (H and I) Proliferation assay (H) and migration assay (I) of F- and M-BMSCs with or without DIC or \( \beta \text{L} \) treatment in the presence or absence of histamine or \( \text{H}_{2}\text{O}_2 \) stimulation, *\( P < 0.05 \).
BMSCs (Figure 1). Our result is consistent with a previous study showing that female muscle-derived stem cells (MDSCs) had higher muscle regeneration efficiency, but failed to observe increased regeneration by normal male MDSCs transplanted into female mice or male MDSCs pre-stimulated with physiological oestrogen levels. These findings together with the results of other studies indicate that sex-related differences might not be exclusively hormonal. Actually, some intracellular signalling pathways and the involved cellular responses to stress were suggested for the explanation about stem cell gender differences. However, the underlying mechanism about the intrinsic difference of stem cell gender remains largely unknown.

The major unexpected finding from the current study was that female BMSCs showed a much higher GAPDH protein level than male BMSCs. Noteworthy, by down-regulating GAPDH in female BMSCs or over-expressing this protein in male cells, the gender differences on inflammatory mediator- and oxidant stress-induced [Ca\(^{2+}\)]i kinetics as well as the cell proliferation and migration were significantly reversed, implying that GAPDH may be the innate molecule which differs the female BMSCs and male BMSCs in the current experiments. Of note, manipulation of GAPDH expression reversed or changed the type of [Ca\(^{2+}\)]i signalling in majority, not all of the cells examined. This may be explained by the heterogeneity of [Ca\(^{2+}\)]i signalling between cells, not by the possible failure of manipulating the GAPDH level in the cells showing no change in the type of [Ca\(^{2+}\)]i signalling, since we manipulated the GAPDH level using stable expression strategy. However, we cannot exclude that the actual GAPDH level is heterogeneous between cells even after manipulating GAPDH expression and there is a threshold level of GAPDH in determining the type of [Ca\(^{2+}\)]i signalling.

Increasing evidence demonstrates that mammalian GAPDH is not merely a simple ‘housekeeping’ protein, but a multifunctional protein with diverse activities instead. GAPDH was involved in the development of various diseases such as Parkinson’s disease, Alzheimer’s disease, and hepatocellular carcinoma. In the present study, the GAPDH level was positively related to cell growth speed and the up-regulation of GAPDH promoted cell proliferation. The results are consistent with a novel finding that GAPDH was implicated in linking glucose availability to the growth and proliferation-promoting mTOR complex. Moreover, our data indicate that GAPDH contributes to cell migration, although little is known about the associated mechanism. Patterson et al. showed that GAPDH physiologically binds to the IP3 R-delivering NADH in close proximity to the channel, and thus regulating intracellular Ca\(^{2+}\). The NAD+/NADH ratio was also shown to modulate ryanodine receptor (RyR)-mediated Ca\(^{2+}\) mobilization. IP3 R-mediated Ca\(^{2+}\) mobilization has been shown to control the growth of human cardiac progenitor cells and their regenerative potential. It contributes to the differentiation of embryonic stem cell-derived cardiomyocytes. RyR-mediated Ca\(^{2+}\) mobilization was also demonstrated to regulate the proliferation and neurogenesis of stem/progenitor cells. Our experiment in vitro demonstrated that sex-related differences of the GAPDH level in BMSCs determined the different pattern of [Ca\(^{2+}\)]i kinetics via the NAD+/NADH ratio. It has been well established in previous investigations including ours that [Ca\(^{2+}\)]i kinetics regulate downstream events such as proliferation and migration. [Ca\(^{2+}\)]i signalling plays a central role in the development of PAH. It is postulated here that the gender difference of GAPDH of BMSCs may regulate BMSCs cellular behaviours including proliferation, migration, and differentiation through [Ca\(^{2+}\)]i signalling and, therefore, determine the therapeutic efficacy against PAH.

Some limitations of this study should be noted. First, the direct link between [Ca\(^{2+}\)], signal kinetics and cellular behaviours needs to be verified in future investigation. Second, former investigators have defined the variability of GAPDH expression between tissues or alterations under pathological conditions like hypoxia. Our results can only highlight the importance of GAPDH on gender differences of BMSCs in basal condition and a pathological circumstance of PAH. Further investigations about GAPDH in pulmonary tissues and particularly in the pathogenesis of PAH become warranted. It has been controversial in the literature about if MCT induced pulmonary hypertension in mice especially in earlier studies. Our results are consistent with the more recent reports showing that MCT induced significant pulmonary hypertension in this type of animals. The reason for the discrepancy remains unclear; however, may be related with the dosage and or the application method of MCT in mice.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

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**References**


32. Tatton NA. Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson’s disease. Exp Neurol 2000;166:29–43.


