Autocrine Activity of Exosomes in Glucocorticoid-Induced Injury of Bone Microvascular Endothelial Cells Identified by Protein Array

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Research

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Abstract

Background: Glucocorticoid could induce injury and apoptosis of bone microvascular endothelial cells (BMECs) in the femoral head and the application of icariin showed a protective effect. However, the impact of autocrine exosomes during these processes is still to be confirmed.

Methods: Exosomes were extracted from BMECs treated with hydrocortisone or hydrocortisone plus icariin by super-speed centrifugation; exosome-carried proteins were evaluated via BCA assay, Western blotting, protein array assay and Elisa test, while miRNA expression profile was assessed via high-throughput sequencing and confirmed by quantitative polymerase chain reaction (qPCR) to screen candidate molecules responsible for BMEC-Exo function. BMECs were incubated with and without exosomes before glucocorticoid intervention and then the impact of BMECs-derived exosomes on BMECs viability, apoptosis, migration, angiogenesis, and protein expression was further assessed by a series of functional assays.

Results: Exosomes secreted by BMECs could ameliorate glucocorticoid-induced endothelial cellular injury, improve cell viability, decrease cell apoptosis, and promote cell migration and angiogenesis compared with the blank control. These effects of secreted exosomes could be reinforced by icariin intervention. Meanwhile, mechanism studies showed that expression level of eNOS, COX-2, and pERK were significantly increased while the cleaved caspase-3 level was decreased in BMECs after coculture with exosomes. Although icariin treatment would not significantly change the size and total protein content of BMECs-derived exosomes, expression of exosome-carried vascular endothelial growth factor (VEGF) and transforming growth factor β 1 (TGF- β 1) was enhanced and numerous miRNAs involved in cell proliferation and apoptosis were up-regulated (e.g., hsa-miR-1469 and hsa-miR-133a-5p) or down-regulated (e.g., hsa-miR-10b-5p) ($p < 0.05$). 29 differentially expressed inflammatory factors were detected between the exosomes secreted by the Icariin-treated and the Model groups.

Conclusion: To sum up, the present study indicates that autocrine exosomes could significantly improve glucocorticoid-induced injury of BMECs, partially mediated by activation of MAPK/ERK pathway and regulation of several inflammation/apoptosis/proliferation-associated proteins. Icariin intervention could reinforce these effects and may act as a promising drug for improving glucocorticoid-induced injury of BMECs. In vivo or animal studies are still required to better understand the function of BMEC-derived exosomes.

Introduction

Synthetic glucocorticoids (GCs) (e.g., prednisolone and prednisone) are a class of anti-inflammatory and immunosuppressive agents widely prescribed in medical conditions like rheumatoid arthritis, bronchial asthma, severe acute respiratory syndrome (SARS), systemic lupus erythematosus, and acute urticaria[1]. Glucocorticoids bind to the glucocorticoid receptors (e.g., NR3C1, GR) on the cell surface, translocate to the nucleus, and target gene transcription, exerting an important role in physiologic function regulation[2].

However, exogenous glucocorticoid administration is also the principal iatrogenic cause for secondary osteoporosis and nontraumatic osteonecrosis of the femoral head (ONFH), which adds a tremendous economic burden on individuals and healthcare system[1, 2]. In patients with glucocorticoid-induced bone loss, angiogenesis is suppressed, accompanied by circulatory impairment, persistent bone destruction, increased apoptosis of osteocytes, disrupted balance of mesenchymal stem cells (MSCs) differentiation, fat embolism, and intramedullary pressure changes[3, 4].

In addition to damaging bone tissues, glucocorticoids have shown a toxic effect on bone microvascular networks[5]. Bone microvascular endothelial cells (BMECs) constitute a monolayer structure attached to bony trabecula, contact with ingredients in the blood, and supply oxygen and nutrients to bone tissues[6]. Moreover, vascular endothelial cells possess secretory functions and can interact with bone cells by secreting angiogenic and osteogenic factors (e.g., Noggin, vascular endothelial growth factor[VEGF]), which is of great significance in the maintenance of bone mass[4, 3]. Last but not least, bone microvasculature provides a specific microenvironment for the colonization of MSCs and other progenitor cells[6]. By elevating the production of hypoxia-induced factor-1 α (HIF-1 α) and reactive oxidative species (ROS), and downregulating expression of endothelial nitric oxide synthase (eNOS), glucocorticoid could lead to injury and apoptosis of vascular endothelial cells, including BMECs[5]. Considering that normally functioning bone vessel networks are a precondition for osseous development and regeneration, BMECs damage is believed to be a common mechanism for glucocorticoid-induced bone diseases[7]. There are multiple bioactive phytochemicals with protective effects on bone strength and vascular function, among which icariin has been widely used to treat osteoporosis and ONFH[8].

Icariin (ICA/C33H40O15) (**Supplementary Figure 1**) is the main active ingredient of Epimedium brevicornum, a traditional Chinese herb known for “strengthening bone and tonifying kidney” for thousands of years[8, 9]. Icariin could promote the proliferation and mineralization of osteoblasts and decrease the activity of osteoblasts[9]. Hu et al. revealed that icariin prevented injury and apoptosis of human umbilical venous endothelial cells following oxidized low-density lipoprotein treatment by regulating apoptosis regulators (caspase 3 and Bcl 2)[10]. Our prior studies demonstrated that intervention of icariin directly ameliorated glucocorticoid-induced injury of BMECs by altering the miRNA and protein expression profiles[11]. However, efforts to understand whether there were other underlying mechanisms are still ongoing.

The updated guidelines of the International Society for Extracellular Vesicles (ISEV) on minimal information for studies of extracellular vesicles (MISEV) provided recommendations on experimental methods and minimal information in reporting and nomenclature of extracellular vesicle (EV)[12]. Exosomes, a population of phospholipid bilayer-enclosed EV ranging from 30 to 100nm in diameter, enable intercellular communication, immunoregulation, tissue regeneration as well as the development of many pathologic conditions through transferring mRNAs, microRNAs (miRNAs), active proteins and other biomolecules between cells, both at autocrine and paracrine levels, at short, medium and long distances. Once stimulated, endothelial cells (ECs) will release exosomes into the blood or extracellular fluid and the function of exosomes originate from ECs are reported to be multi-faceted: on the one hand, they

contribute to the onset of cardiovascular disorders (e.g., atherosclerosis) and on the other hand, may enhance endothelial survival[13]. In recent years, it has been confirmed that the autocrine activity of exosomes could promote the developments of multiple pathological changes such as diabetes mellitus 2, glioblastoma and ischemic diseases[14, 15].

Based on the results of previous studies, we aimed to investigate the role of autocrine activity of ECs-derived exosomes in the development of glucocorticoid-induced injury of BMECs, angiogenesis and icariin intervention. Fractures associated with glucocorticoid-induced osteoporosis usually occur in regions with massive cancellous bone, especially the spine and proximal femur; meanwhile, osteonecrosis predominantly arises in the femoral head[16]. Given that ECs in different regions manifest morphological and functional specificity, BMECs isolated from the femoral head were used to conduct the current investigation.

Materials And Methods

This experimental protocol was designed following the World Medical Association Declaration of Helsinki on Ethical principles for medical research involving human subjects and approved by the Ethics Committee of China-Japan Friendship Hospital. The inclusion criterion was patients diagnosed as primary osteoarthritis (OA) of the hip, osteoarthritis secondary to developmental dysplasia of the hip (DDH) as well as femoral neck fracture, and subjected to total hip arthroplasty. Written informed consent must be signed before surgery. Eventually, BMEC isolated from a total of eighteen donors (mean age 59.9 ± 9.0 years, range: 43-64; F/M: 4/4; six primary OA, six OA secondary to DDH and six femoral neck fractures) admitted to the orthopedic department of China-Japan Friendship Hospital from May 2017 to January 2021 were used to conduct experiment presented herein.

BMECs isolation

Normal cancellous bones from resected femoral heads were cut into approximately 1-mm³ particles (Figure 1A & B) and BMECs were isolated following methods described in the previous article[17]. The purity of extracted BMECs was evaluated through immunofluorescent staining.

CCK-8 assay

BMECs were seeded in 96-well plates at 5×10^3 /well, cultured in complete medium for 12 h, and treated with icariin (Solarbio Science and Technology Co. Ltd., China; purity $\geq 98\%$) in different concentrations (0 M, 1×10^{-6} M, 1×10^{-5} M, 5×10^{-5} M and 1×10^{-4} M) for another 24 h. Icariin was dissolved in DMSO, whose final concentration in the organ bath solution did not exceed 0.1% and therefore would not cause a significant effect on the survival and proliferation of endothelial cells[18]. Then BMECs were treated with hydrocortisone (0.3 mg/mL) (R&D Systems, UAS) for another 24 h. 10 μ l of CCK8 reagent was added to 90 μ l of culture medium per well and incubated for 4 h. Subsequently, the optical density (OD) at 450 nm was detected using a microplate reader to calculate the cell proliferation.

Exosome extraction and identification

After adherence, BMECs were cultured in an exosome-free medium (prepared by filtration through a 0.22µm filter and then centrifugation at 100,000 × g for 18 min) with and without icariin for 24 h. Then the supernatant was collected to extract exosomes using the exosome isolation kit (MagCapture™ Exosome Isolation Kit PS, Japan). Each group of exosomes was extracted from an equivalent number of BMECs and resuspended in PBS; their diameter distribution was analyzed using nanoparticle tracking analysis (Nanosight NS300, England). The exosome proteins were assessed by Western blot assay using primary antibodies to CD9 (Santa Cruz Biotechnology; dilution 1:1000) and CD81 (Santa Cruz; dilution 1:800).

High-throughput sequencing transcriptome and quantitative polymerase chain reaction (qPCR) of exosome-carried miRNAs

After extracting RNA from exosomes by using TRIzol reagent, high-throughput sequencing of exosome-carried miRNAs was performed according to standard procedures provided by Illumina. A small RNA sequencing library was prepared using the TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA). Then the constructed library was sequenced using Illumina Hiseq 2000/2500 platform. MiRNAs with lower expression (those with less than 10 copies in all samples) were first removed from the analysis. Then differentially expressed genes between BMECs in the icariin-treatment and the non-treatment groups were screened using $|\log_2(\text{Fold change})| > 1$ and $p\text{-value} \leq 0.05$ as the threshold. Then, genes related to proliferation, angiogenesis and apoptosis of endothelial cells were selected for confirmation by using qPCR. The reverse transcription process was performed using the One-Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Japan) and the amplification reaction was conducted using the SYBR® Premix Ex Taq™ II kit (Takara, Japan).

Bicinchoninic acid (BCA) analysis

Total proteins were extracted from exosomes and measured using a BCA protein assay kit (Sigma, St. Louis, MO, USA). 200 µl of BCA working solution was added to 10 µl of extracted protein sample per well of 96-well plates and incubated in the dark for 30 min at 37°C. The OD value was measured at 750 nm, and the protein concentration was calculated from the standard curve, with bovine serum albumin as the standard protein.

Antibody array assay and Elisa detection

The isolated proteins were scanned by using the GSH-CAA-440 array to obtain the raw data. Then the Raybiotech software was used to remove the chip background and normalize the original data. Differentially expressed proteins (DEPs) between different groups were identified using the limma package (Version 3.42.2) in R software. Individual p -values were calculated and converted to adjusted p -values (adj. p . val) for comparisons by false discovery rate correction of the Benjamini and Hochberg test.

A cutoff point of adj. *p. val* < 0.05 and |fold change (FC)| > 1.2 were used to select DEPs. Then the heatmap, PCA plot and volcano plot of the DEPs were drawn using the ggplot2 package in R software. The expression levels of specific protein of interest were determined by ELISA.

Functional enrichment analyses and annotation of DEPs

The DEPs were uploaded to an online bioinformatics database, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 Beta (<https://david-d.ncifcrf.gov/>), for enrichment analysis including gene ontology (GO) and Kyoto Encyclopedia of Gene and Genome (KEGG) pathways. The obtained results were visualized in the R ggplot2 package and a *p*-value of less than 0.05 was considered statistically significant.

The protein-protein interaction (PPI) network was constructed by using the multiple protein online tool in STRING database (version 11.0, <http://string-db.org>)[19] and visualized in Cytoscape software (Version 3.6.2). The targeted TFs and miRNAs of DEPs were predicted using the NetworkAnalyst platform (<https://www.networkanalyst.ca/faces/home.xhtml>).

Coculture of BMECs and exosomes

According to the intervention condition, BMECs were divided into three groups: in the Icariin-BMEC-Exo group, BMECs were cultured in the presence of exosomes derived from BMECs treated with icariin; in the BMEC-Exo group, BMECs were co-cultured with exosomes derived from BMECs without icariin treatment and in the control group, BMECs were free from exosomes intervention. In the former two groups, the exosome concentration was adjusted to 200 ng/μl. After 24 h of cell culture, all three groups were treated with hydrocortisone.

Cell scratch assay

BMECs were inoculated in 6-well plates and when cells reached confluence as a monolayer, a 1 mL pipette tip was used to scratch the cell layer following center lines of wells gently. Exosome and hydrocortisone (0.3 mg/mL) intervention was performed following steps described before and after 12 h and 24 h, images were taken under an inverted microscope.

Tube formation assay

BMECs with and without exosome intervention were inoculated to 24-well plates coated with enriched growth factor Matrigel (BD Biosciences, USA) for 24 h and then treated with hydrocortisone (0.06 mg/mL). After 4 and 8 h of hydrocortisone intervention, images were taken under the inverted microscope (Olympus, Japan).

Flow cytometry assay

After 12 h of hydrocortisone (0.3 mg/mL) intervention, BMECs were harvested and washed twice with PBS at 4°C and resuspended with 300 μl of binding buffer. 100 μl of cell suspension, 5 μl of AnnexinV-

FITC and 10 μ l of propidium iodide (PI) solution (20 ng/L) were mixed at room temperature, incubated in the dark for 15 min and then the fluorescence intensity was detected by using a flow cytometer.

Western blotting

After 24 h of hydrocortisone (0.3 mg/mL) intervention, Western blotting was performed. Proteins of interest were listed as follows: extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphorylated ERK1/2 (pERK1/2), cyclin-dependent kinase inhibitor 2C (CDKN2C), endothelial nitric oxide synthase (eNOS), cyclooxygenase-2 (COX-2), caspase-3 and cleaved caspase-3.

Statistical analysis

Data are presented in the form of mean $\pm$ standard deviation (SD). The differences between groups were analyzed by the Student's *t*-test and one-way analysis of variance (ANOVA). A $p < 0.05$ indicates a statistically significant difference. All statistical tests were performed using prism statistical software (version 6.0, San Diego, USA).

Results

Cell morphology observation and identification

After 3 days of cell culture, it could be observed that adherent cells were evenly distributed in culture bottles (Figure 1C). When isolated cells grew to 80% confluence, they became fusiform or polygonal, and presented a cobblestone-like appearance under the inverted-phase contrast microscopy (Figure 1D). Under the transmission electron microscope, these cells were in polygonal shape with large ovular nuclei of obvious nucleoli and the surface was decorated with microvilli formed by cytoplasm bulge; cytoplasm was rich in mitochondria, ribosomes, rough endoplasmic reticulum, Golgi complexes and endothelial-specific Weibel-Palade bodies (Figures 1E & 1F). Immunofluorescent staining of isolated cells revealed high positive rates for vWF and CD31, and a high negative rate for CD133 (all nearly 100%), indicating that these isolated cells were BMECs (Figures 1G, 1H & 1I).

Effects of icariin on cell proliferation rate

Compared with the icariin-free group, the proliferation rate of BMECs increased as the concentration of icariin increased ($3.98\% \pm 1.76\%$, $7.04 \pm 1.07\%$, $10.10 \pm 0.83\%$, $29.86 \pm 0.75\%$ and $31.96 \pm 1.75\%$ in the 0 M, 1×10^{-6} M, 1×10^{-5} M, 5×10^{-5} M and 1×10^{-4} M groups, respectively) (Figure 2A). The proliferation promotion effects of icariin in the 5×10^{-5} M and 1×10^{-4} M groups were most significant with no statistical differences ($p > 0.05$). So, 5×10^{-5} M was chosen as the optimal concentration for subsequent experiments.

Size of extracted exosomes

In the control group, the average diameter of exosomes was 86.68 nm, while in the icariin-treated group, the average diameter of exosomes was 85.35 nm. We found that icariin treatment did not significantly alter the diameter of exosomes derived from BMEC cells in two groups.

Protein analysis of extracted exosomes

The BCA results showed that the exosome protein content was 132.63 ± 3.33 $\mu\text{g/mL}$ in the control group and 138.56 ± 9.58 $\mu\text{g/mL}$ in the icariin-treated group. The difference in exosome-carried protein concentration between the two groups was not statistically significant. CD9 and CD81, two representative exosome surface proteins, were highly expressed in EVs extracted from cells of both the control group and the icariin-treated group (Figure 3B), indicating that isolated EVs were indeed exosomes. Furthermore, the expression of VEGF and TGF- β 1 protein was elevated in exosomes derived from cells in the icariin-treated group compared with those from the control group ($p < 0.01$) (Figure 3B).

MiRNA high-throughput sequencing and qPCR

A total of 50 differentially-expressed miRNAs between the icariin-treated and the control groups was identified, among which 20 miRNAs were down-regulated and 30 miRNAs were upregulated after icariin treatment (**Supplementary table 1**). Exosome-derived miRNAs were reported to affect the angiogenesis of receipt cells. In this study, 2 downregulated miRNAs (hsa-miR-133a-5p and hsa-miR-10b-5p) and 1 downregulated miRNA (hsa-miR-1469) were considered to potentially play important roles in the protective effect of icariin to glucocorticoid-induced injury of BMECs. Hsa-miR-133a-5p may protect against hypoxia/reperfusion-induced or ischemia/reperfusion-induced hepatocyte injury by decreasing expression of MARK6[20], while upregulation of hsa-miR-10b-5p could inhibit caspase-3 activation and therefore prevent cell apoptosis[21]. Conversely, hsa-miR-1469 has been demonstrated to induce apoptosis in a series of cancer cells[22]. The difference of expression level of these three miRNAs between Icariin-BMEC-Exo and BMEC-Exo was further confirmed by qPCR ($p < 0.01$) (Figure 3C). The primer sequences of investigated miRNAs were: hsa-miR-1469, forward, 5'-GCGCAGCTGGTAAATGGAA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; hsa-miR-133a-5p, forward, 5'-GCCTCGGCGCGGGGCGCG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; hsa-miR-10b-5p, forward, 5'-GCTACCCTGTAGAACCGAA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'.

Data normalization and DEPs identification

Data normalization and cross-comparability were evaluated using the principal component analysis (PCA) for confirming biological variability between different samples (Figure 4A). In total, 62 DEPs were identified between the Model group and the control group, including 11 upregulated and 51 downregulated DEPs, which were listed in **Supplementary Table 2**. Meanwhile, 29 DEPs were detected between the Icariin-treated group and the Model group, including 25 upregulated and 4 downregulated DEPs. In addition, a volcano plot and a heatmap of all DEPs between the Icariin-treated and the Model groups were generated using the R ggplot2 package (Figure 4B & 4C). Elisa assay revealed confirmed the expression level of five DEPs including EMMPRIN, Galectin-3, ICAM-1, MIF and MMP-3 (Figure 2D).

GO function and KEGG enrichment analysis of DEPs

GO enrichment analysis of 29 DEPs between the Icariin-treated group and the Model group was performed to identify the most relevant biological processes (BPs), molecular functions (MFs), and cellular components (CCs). The top ten enriched terms in BPs, CCs, and MFs were presented in Figures 3D. Additionally, based on KEGG pathway analysis, the DEPs were significantly enriched in 3 signaling pathways, such as cytokine-cytokine receptor interaction (8 proteins), viral protein interaction with cytokine and cytokine receptor (4 proteins), and TNF signaling pathway (3 proteins) (Figure 3D).

PPI network construction and hub gene selection

The interactions between the proteins expressed from DEPs, which consisted of 24 nodes and 35 edges (**Supplementary Figure 2A**), were constructed from the STRING database and visualized using Cytoscape. In addition, 9 of the top genes with connectivity degrees ≥ 3 were: ICAM1 (Degree = 11), MMP3 (Degree = 8), BSG (Degree = 5), LGALS3 (Degree = 5), CXCL9 (Degree = 4), CXCL16 (Degree = 4), MMP10 (Degree = 3), IL36RN (Degree = 3), and TNFRSF1B (Degree = 3). The top 9 hub genes were also selected by CytoHubba based on the MCC and MNC classification methods (Table 1), showing overlapping genes with different priorities.

Table 1
List of the top 9 hub genes selected by
MCC, MNC and degree methods in
cytoHubba

MCC	MNC	Degree
ICAM1	ICAM1	ICAM1
MMP3	MMP3	MMP3
BSG	BSG	BSG
LGALS3	CXCL9	LGALS3
CXCL9	CXCL16	CXCL9
CXCL16	LGALS3	CXCL16
MMP10	MMP10	MMP10
IL36RN	IL36RN	IL36RN
TNFRSF1B	TNFRSF1B	TNFRSF1B

TF-miRNA interacted network

The TF-miRNA interacted network was constructed through network analysis of 29 DEPs in Cytoscape (**Supplementary Figure 2B**), which included 27 genes, 27 TFs, and 15 miRNAs, involving 99 associations between the TFs and DEPs and 34 associations between the miRNAs and DEPs. We separately analyzed

the degree of 27 DEPs in the TF-DEP network and the microRNA-DEP network (Table 2). Then, we found that MYC and SP1 regulated 7 interacting DEPs. Simultaneously, hsa-miR-17 regulated 3 interacted DEPs.

Table 2
Transcription factors-miRNA-gene interacted network.

MiRNAs	Targeted genes	Gene counts	Transcription factors (TF)	Targeted genes	Gene counts
hsa-miR-330-3p	DKK3, MME, WIF1, TNFRSF1B, IL36RN	6	MYC	ICAM1, BSG, ICAM2, FTL, LGALS1, CSTB, ENO2	7
hsa-miR-17	ICAM1, MMP3, SIGLEC10, RGMB	5	SP1	ICAM1, ICAM2, FTL, CSTB, SIGLEC10, LGALS9, MME	7
hsa-miR-22	FTL, LGALS1, BMP7	3	NFKB1	ICAM1, ICAM2, MMP3, GH1, FSTL3, TNFRSF1B	6
hsa-let-7d	DKK3, LYVE1	2	MAX	ICAM, BSG, ICAM2, IGFBP6, CSTB, TNFRSF1B	6
hsa-miR-106a	MMP3, RGMB	2	JUN	ICAM1, MMP3, FTL, CSTB, LGALS3, CXCL16	6
hsa-miR-124	LRP6, FSTL3	2	SPI1	ICAM1, FTL, IGFBP6, SIGLEC10, GH1, MME	6
hsa-miR-129-5p	LRP6, WIF1	2	CTCF	LGALS1, MMP10, BMP7, TNFRSF1B, WIF1, RGMB	6
hsa-miR-19a	LRP6, TNFRSF1B	2	RELA	ICAM1, ICAM2, MMP3, FSTL3, TNFRSF1B	5
hsa-miR-216b	MME, WIF1	2	TFAP2A	BSG, CSTB, TIMP4, SIGLEC10	4
hsa-miR-32	DKK3, MMP10	2	FOS	MMP3, FTL, LGALS3, TNFRSF1B	4
hsa-miR-329	DKK3, FSTL3	2	TBP	MMP3, FTL, MMP10, GH1	4
hsa-miR-577	MMP3, LRP6	2	TFAP2C	BGS, LGALS3, TIMP4	3
hsa-miR-708	DKK3, CXCL9	2	STAT1	ICAM1, CXCL9, TNFRSF1B	3
hsa-miR-9	MME, LYVE1	2	EBF1	LGALS1, LGALS3, GH1	3

Effect of BMEC-derived exosomes on BMEC function

Effect of exosomes on endothelial cell migration and tube formation

After 12 h and 24 h of hydrocortisone treatment, the migration ability of BMECs pretreated with exosome was significantly higher than those without exosome treatment ($p < 0.05$) (Figures 4A & 4B). Exosome pretreatment could also increase endothelial cells' angiogenic capacity, including the number of branch points, the number of cavities and tube length ($p < 0.05$). The ability to promote migration and angiogenesis of microvascular endothelial cells was more pronounced in the Exo-Icariin-BMECs group in comparison with the Exo-BMECs group ($p < 0.05$) (Figures 4C, 4D & 4E).

Cell viability

After treatment with hydrocortisone (0.3 mg/mL) for 0, 1, 2, 3, and 4 days, the viability of BMECs in the BMEC-Exo group and Icariin-BMEC-Exo group was significantly enhanced in comparison with that of the control group ($p < 0.01$). This effect was dominant in the Icariin-BMEC-Exo group compared with the BMEC-Exo group ($p < 0.01$) (Figures 5A & 5B).

Cell apoptosis rate

After being treated with 1 mg/mL of hydrocortisone for 12 h, apoptotic rates of BMECs in the BMEC-Exo group and Icariin-BMEC-Exo group were lower than those in the control group ($p < 0.01$). Similarly, the inhibitory effect for apoptosis is more significant in the Icariin-BMEC-Exo group compared with the BMEC-Exo group ($p < 0.01$) (Figure 5C). These results indicated that the protective effect of icariin on the glucocorticoid-induced injury of BMECs was partially realized via autocrine secretion of BMECs-derived exosomes.

Western blotting analysis

There were no significant differences in the expression level of ERK1/2 in the three groups. However, compared with the control group, upregulated pERK, VEGF, eNOS, COX-2 and procaspase-3 were identified in the BMEC-Exo group and the Icariin-BMEC-Exo group in comparison with the control group, accompanied by the downregulated level of cleaved caspase 3 and CDKN2C (all $p < 0.01$). Likewise, the amount change of these proteins is more significant in the Icariin-BMEC-Exo group compared with the BMEC-Exo group (all $p < 0.01$) (Figure 5D). These results suggested that exosomes could be engulfed by receipt cells and therefore transduce signals to multiple downstream pathways. MARK/ERK has been shown to exert a pivotal role in VEGF-induced endothelial cell proliferation as well as the regulation of cell differentiation, apoptosis and angiogenesis[23]. Besides, nitric oxide (NO) and endothelial prostacyclin (prostaglandin I₂; PGI₂) are both important vasodilators and mediators of ECs functions; while eNOS and COX-2 act as key enzymes in the formation of NO and PGI₂, respectively[24]. CDKN2C, a member of the INK4 family of cyclin-dependent kinase inhibitors, has been revealed to interact with CDK4 or CDK6, prevent the activation of the CDK kinases and serve as a cell growth regulator inhibiting cell cycle G1 progression[25]. Meanwhile, apoptotic signaling pathways of vascular endothelial cells exercise their

functions by activating caspase-3 and it is reported that application of caspase-3 inhibitor can significantly reduce oxidized low-density lipoprotein-induced apoptosis[26].

Discussion

By using bone microvascular endothelial cells extracted from the femoral head, the experimental conditions are closer to the microvasculature in bone than those using human umbilical vein endothelial cells. The preceding finding has revealed that a hydrocortisone concentration of more than 0.3mg/mL would induce typical proliferation inhibition and apoptosis of BMECs[11]. In this study, the extracted extracellular vesicles exhibited classic morphological and biochemical features of exosomes, and we demonstrated that autocrine activity of exosomes ameliorated glucocorticoid-induced apoptosis of BMECs as well as improved cell viability, migration and angiogenesis. Loads of evidence investigated the clinical application of icariin, in which dosage of icariin generally varied from 5×10^{-6} M to 5×10^{-5} M[27]. The present study has shown that exposure to icariin could remarkably enhance BMECs viability in a dose-dependent and dose-saturable manner and based on the results of CCK-8 assay, and 5×10^{-5} M was identified as the optimal concentration for promoting BMECs viability and reinforcing the protective effect of autocrine exosomes for glucocorticoid-induced injury of BMECs. This effect was partially realized by changing protein and RNA profiling of secreted exosomes of bone microvascular endothelial cells.

BMECs-derived exosomes, defined as exosomes released by BMECs during external activation, might be initiated by chemical (e.g., TNF α and IL-6) and physical (e.g., shear stress) factors[28]. Responses of endothelial exosomes to various medications are demonstrably different. Zhao and his colleagues[29] shown that lipopolysaccharide increased the protein quantity of exosomes released from pulmonary artery ECs, which further enhanced proliferation and prevented apoptosis of pulmonary smooth muscle cells. TNF- α would not influence endothelial exosome concentration, but might alter exosome-carried protein and miRNA composition. For BMECs, icariin treatment significantly changed the size and total protein level of secreted exosomes, but could alter the composition of contents packaged in exosomes, dramatically increasing the amounts of VEGF, TGF- β 1, hsa-miR-133a-5p, and has-miR-10b-5p in exosomes while leading to lesser hsa-miR-1469. VEGF and TGF- β 1 are often co-expressed in tissues and considered to be prominent promoters of angiogenesis; however, they show opposite effects for ECs[30]. The upregulated expression level of VEGF can be initiated by hypoxia, multiple cytokines and growth factors, including TGF- β 1. After combining with VEGF receptor 2 (VEGFR2), VEGF further transduces signals to downstream molecules (e.g., ERK1/2, c-Jun N-terminal kinase/JNK, phosphatidylinositol 3 kinase/PI3K, AKT, eNOS and P70S6K), inhibits the apoptosis of ECs and modulates a series of other physiological functions[31]. On the other hand, the possible mechanisms of TGF- β 1 for enhancing angiogenesis and differentiation of ECs is manifold[32]. First, the optimal level of ECs apoptosis is a precondition for the formation of a functional vascular network, and inhibition of apoptosis may lead to abnormality of angiogenesis. Second, TGF- β 1 could increase the expression of VEGF and other angiogenic factors in ECs. Third, ECs apoptosis induced by TGF- β 1 is a transient process, occurring

rapidly and followed by a prolonged refractory period. Last but not least, it has been indicated that TGF- β 1 promoted angiogenesis through the regulation of immune cells in vivo.

Moreover, to systematically identify novel inflammatory cytokines related to the protective effect of icariin, we performed a screen among the Model group, Icariin-treated group and the control group by protein array assay. The present study found that 29 proteins were dysregulated in exosomes secreted by BMECs after icariin intervention and most of these proteins were upregulated. Based on the PPI network, 9 proteins (ICAM1, MMP3, BSG, LGALS3, CXCL9, CXCL16, MMP10, IL36RN, and TNFRSF1B) with high connectivity were identified to play essential roles in the protective effect of icariin to glucocorticoid-induced injury of BMECs. ICAM1 is a cell surface glycoprotein typically expressed on endothelial cells[33]. Meanwhile, ICAM1 might exert dual effects on bone homeostasis, especially in osteoclastogenesis[34]. MMP3 and MMP10 belong to the matrix metalloproteinase family and also participate in osteogenic differentiation[35]. LGALS3, which is widely expressed in various tissues including bone tissues, is considered as a marker of bone formation[36]. Important miRNAs (e.g., hsa-miR-330-3p and hsa-miR-17) and transcription factors (e.g., MYC and SP1) may involve in the dysregulation of these proteins. In former studies, these molecules have been demonstrated to be vital for vascular development and angiogenesis [37, 38]. Cytokine-cytokine receptor interaction pathway is predicted to be significantly enriched by identified DEPs, which included CCL14, TNFRSF10C, CXCL9, IL36RN, BMP7, TNFRSF1B, CXCL16, and GH1.

ECs-derived exosome affects recipient cells' function and modulates key events in the development of multiple disorders; the effect could be either beneficial or detrimental. It has also been shown that capillary endothelial tip cells secrete exosomes incorporating Delta-like 4 ligands, which are passed to tip cells of endothelial sprout, promote cell motility and suppress their proliferation[39]. Senescent human ECs-derived exosomes could knock down Frizzled-3 of human MSCs and inhibit osteogenic differentiation[40]. Our study demonstrated that exosome intervention resulted in upregulated production of angiogenic (e.g., VEGF, pERK) and vasodilating factors (e.g., eNOS and COX-2), while inhibited the activation of pro-apoptotic factors (cleaved caspase-3) in endothelial cells, indicating that endothelium-dependent signaling pathways might involve in the protective effect of autocrine exosomes to glucocorticoid-induced endothelial cellular injury. Apoptosis is a kind of programmed cell death occurring in a multicellular organism to maintain homeostasis, with caspase-dependent apoptosis being demonstrated to be the most classical mechanism of glucocorticoid-induced endothelial cell death[41]. BMECs-derived exosomes intervention significantly inhibited the cleavage of pro-caspases-3 and therefore improved the apoptosis of BMECs. NO is synthesized by eNOS using amino acid L-arginine and is able to regulate vascular tone[18]. Inhibition of the VEGF/Akt/eNOS pathway blocks nitric oxide release and promotes vasoconstriction. As for cyclooxygenase, there are two isoforms discovered currently: COX-1 and COX-2, with the latter one being the rate-limiting key enzyme in the biosynthesis of prostaglandins during inflammatory responses. A complex interplay between eNOS and COX-2 pathways can be observed in the relaxation of vascular smooth muscles[42]. ECs-derived exosome intervention may lead to enhanced NO and PGs production in vivo conditions; although eNOS and COX-2 inhibitors did not impact the viability of endothelial cells in rescue experiments, whether these factors ameliorate apoptosis

and migration remains to be confirmed. In our studies, exosomes pretreatment promoted the level of the phosphorylated extracellular regulated protein kinases, the active form of ERK, revealing that BMECs-derived exosomes improve glucocorticoid-induced BMEC injury partially via activation of ERK pathways. Among four classical mitogen-activated protein kinases (MAPK) signaling pathways, MAPK/ERK pathway is reported to be associated with regulation of proliferation, survival, apoptosis and angiogenesis of endothelial cells[23].

This study is not without limitations. First, extrapolation of results from cell models to clinical practice should be performed with caution. It is needed to consider the impact of factors in vivo. Second, although hsa-miR-133a-5p and hsa-miR-10b-5p were enriched after icariin intervention, we did not perform the further experiment to test whether they could take effects in the reversion of glucocorticoid-induced cellular injury. Last but not least, it is well-acknowledged that icariin could affect multiple cell types existing in bones, for example, MSCs and osteocytes; meanwhile, exosomes can modulate inflammation, regulate activation and migration of monocytes as well as affect differentiation of MSCs. Therefore, it is rational to assume that other kinds of cells in the bone or even cells of cardiovascular systems in the distance may also engulf BMECs-derived exosomes may also be engulfed by other kinds of cells. In vivo and animal studies are required to confirm these findings and further illuminate the action mechanism of identified miRNAs and proteins for the protective effects of icariin to glucocorticoid-induced injury of BMECs.

Conclusion

The present study indicates that autocrine exosomes could significantly improve glucocorticoid-induced injury of BMECs, partially mediated by activation of MAPK/ERK pathway and regulation of several apoptosis/proliferation-associated proteins. Icariin intervention could reinforce these effects and may act as a promising drug for improving glucocorticoid-induced injury of BMECs. In vivo or animal studies are still required to gain a better understanding of the function of BMEC-derived exosomes.

Abbreviations

BMECs: bone microvascular endothelial cells; ISEV: International Society for Extracellular Vesicles; EV: extracellular vesicles; eNOS: endothelial nitric oxide synthetase; COX-2: cyclooxygenase-2; pERK: phosphorylated extracellular regulated protein kinases; VEGF: vascular endothelial growth factor; TGF- β 1: transforming growth factor β 1; SARS: severe acute respiratory syndrome; ONFH: nontraumatic osteonecrosis of the femoral head; MSCs: mesenchymal stem cells; ROS: reactive oxidative species; PGI₂: prostaglandin I₂; miRNAs: microRNAs; ECs: endothelial cells; BCA: Bicinchoninic acid; qPCR: quantitative polymerase chain reaction; SD: standard deviation; ANOVA: one-way analysis of variance; ECVs: extracellular vesicles; VEGFR-1 and -2: vascular endothelial growth factor receptor 1 and 2; MAPK: mitogen-activated protein kinases

Declarations

Ethics approval and consent to participate

This study was performed in conformity with the Helsinki declarations and all protocols were approved and monitored by the Institutional Review Board of the China-Japan Friendship Hospital (Ethical commission Number 2018-GZR-100). All participants provided informed written consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Authors' contributions

WS and FQG designed the study. QYZ, TQL, XJW and FQG contributed to the experimental work. QYZ and TQL prepared the first draft of the paper. JKL, FQG, WS and ZRL critically revised the manuscript. QYZ, TQL, JKL, XJW, FQG and WS were responsible for statistical analysis of the data. All authors revised the paper critically for intellectual content and approved the final version. All authors read and approved the final manuscript.

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Figures

Figure 1

Obtaining of tissue sample, cell morphology observation and purity identification. A) Longitudinal section of the femoral head after femoral neck fracture; B) bone granules for further extraction of cells; C) three days after cell isolation, adherent cells evenly distributed in the bottom areas of the culture dishes, and were an inconsistent in size and shape (100× magnification); D) isolated cells shows cobblestone morphology after 7 days of culture (40× magnification); E & F) under scanning electron scope, cells were covered with microvilli and rich in organelles; G & H & I) immunofluorescent staining shown high positive rates of CD31 (G) and vWF (H) as well as high negative rate of CD133 (I).

Figure 2

Icariin treatment and analysis of package in exosome. A) Proliferation rate of BMECs increased with the concentration of icariin; effects of icariin in the 5×10^{-5} M and 1×10^{-4} M groups are most significant with no statistical differences; B) protein expression bands of VEGF, TGF- β 1, CD9 and CD81 in the icariin-treated group and the control group; C) the expression level of hsa-miR-1469, hsa-miR-133a-5p and hsa-miR-10b-5p in exosomes extracted from the icariin-treated group and the control group measured by

using qPCR; D) relative expression level of hub proteins identified by Elisa assay. HYD indicated hydrocortisone (0.3 mg/mL). Data analysis was performed using a t-test, error bars indicate standard deviation, and ** means $p < 0.01$, * means $p < 0.05$.

group; (E). The most relevant biological processes (BPs), molecular functions (MFs), and cellular components (CCs) through GO enrichment analysis of differentially expressed proteins between the Icariin-treated group and the Model group.

Figure 4

Effect of exosomes on endothelial cell migration and angiogenesis. A) Observation of scratch assay at 0, 12, and 24 h after hydrocortisone treatment; B) scratch closure rate in three groups; C) tube formation assay at 4 h and 8 h after hydrocortisone treatment; D & E) number of branch points, number of cavities and tube length at 4 h and 8 h after hydrocortisone treatment. Data analysis was performed by one-way analysis of variance, with error bars indicating SD, ** indicating $p < 0.01$, and * indicating $p < 0.05$. Control indicates BMEC that have not been co-cultured with exosomes but treated with hydrocortisone, BMEC-Exo

represents BMEC that have been co-cultured with exosomes secreted by bone microvascular endothelial cells without icariin intervention, and Icariin-BMEC-Exo indicates BMEC that have been co-cultured with exosomes secreted by bone microvascular endothelial cells treated with icariin and then treated with hydrocortisone.

Figure 5

Co-culture with exosomes improved the viability and decreased apoptosis of BMECs. These effects are more pronounced in Icariin-BMEC-Exo group than in BMEC-Exo group. (A) Detection of OD values at various time points after hydrocortisone intervention; (B) cell viability at each time point; (C, D) cell apoptosis detected by flow cytometry; (E) the expression level of multiple proteins in different groups of BMECs. Control indicates BMEC that have not been co-cultured with exosomes but treated with hydrocortisone, BMEC-Exo represents BMEC that have been co-cultured with exosomes secreted by bone microvascular endothelial cells without icariin intervention, and Icariin-BMEC-Exo indicates BMEC that have been co-cultured with exosomes secreted by bone microvascular endothelial cells treated with icariin and then treated with hydrocortisone. Data were analyzed using two-way analysis of variance or one-way analysis of variance, error bars indicate standard deviation, and ** indicates $p < 0.01$.

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