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Original article

Metformin pre-conditioning enhances the angiogenic ability of the secretome of dental pulp stem cells

Nezar Boreak [a], Noor Mohammed Abu Khayrat [b], Ahmed Omar Shami [b], Haitham Jameel Mohammed Zaylaee [b], Aeshah Ali Hanbashi [b], Sara Abdulhamed Souri [b], Hala Mohammed Otayf [b], Rafif Essam Bakri [b], Mohammad Eissa Mansoor Ajeely [b], Alwaleed Essam Hassan Bakri [b], Mohammed Abdurabu Jafer [c,d], A. Thirumal Raj [e], Hosam Ali Baeshen [f], Shankargouda Patil [g,*]

[a] Department of Restorative Dental Sciences, College of Dentistry, Jazan University, Jazan, Saudi Arabia
[b] College of Dentistry, Jazan University, Jazan, Saudi Arabia
[c] Department of Preventive Dental Science, College of Dentistry, Jazan University, Saudi Arabia
[d] Department of Health Promotion, Maastricht University/CAPHRI, The Netherlands
[e] Department of Oral Pathology and Microbiology, Sri Venkateswara Dental College and Hospital, Chennai, India
[f] Department of Orthodontics, College of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia
[g] Department of Maxillofacial Surgery and Diagnostic Sciences, Division of Oral Pathology, College of Dentistry, Jazan University, Jazan, Saudi Arabia

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ABSTRACT

The aim of the present study was to assess the influence of metformin on the angiogenic ability of secretomes from dental pulp stem cells. The stem cells were obtained from the dental pulp (DPSCs) (n = 3) using the explant culture method. We treated the DPSCs with different concentrations of metformin and assessed the expression of the angiogenesis-related genes. We also tested the angiogenic effect of the secretomes on the yolk sac membrane of the chick embryos by counting the quaternary blood vessel formations on the yolk sac membrane. We found that metformin treatment enhanced the angiogenic potential of the stem cell secretome in a dose-dependent manner. This was evidenced by the increase in the quaternary blood vessel formations in the yolk sac membrane with lower to higher concentrations of metformin. Pre-treatment with metformin modulates the angiogenic potential of the stem cell-conditioned media in a dose-dependent manner. The augmentation of the angiogenic potential of the DPSCs can aid regeneration, especially in scenarios requiring the regeneration of vacuoles.
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1. Introduction

The primary function of the dental pulp is to sustain homeostasis of the teeth (Rena et al., 2017). Pulps are frequently vulnerable to various disturbances. The homeostasis of the dental pulp can be disrupted by injury and inflammation secondary to damage, which can adversely disturb the restoration of tissue in this critical niche deficient in blood supply (Gharaei et al., 2018). Regenerative dentistry is an emerging field of the dental and oral sciences that aims to restore injured/missing dental pulp tissue by employing the fundamentals of cellular and molecular biology, rather than just conservative treatment modalities in endodontics (Kim et al., 2015). Mesenchymal stem cells (MSCs) are important for mesodermal, endodermal, and ectodermal lineages (Rodríguez-Lozano and Moraleda, 2011). MSCs have been isolated from several tissues, including bone marrow, adipose tissue, umbilical cord, and oral tissues such as dental pulp MSCs (DPSCs) (Raj et al., 2021; Rodríguez-Lozano et al., 2012; Ducret et al., 2015). Recent studies have shown promising outcomes of the augmentation of the regenerative potential of DPSCs using biomolecules. Melatonin strongly influences the immunomodulatory properties of DPSCs (García-Bernal et al., 2021). Cobalt chloride-induced hypoxia promotes DPSC survival and alters its secretome profile and stemness (Bhandi et al.,

* Corresponding author.
E-mail addresses: drbaeshen@me.com (H.A. Baeshen), dr.ravipatil@gmail.com (S. Patil).
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2021). The dental pulp tissue is highly vascularized, which is largely attributed to the remarkable intrinsic regenerative capability (Di Pietro et al., 2015). Angiogenesis is vital for the regeneration and development of the dentin-pulp complex (Bakhashab et al., 2016). Angiogenesis creates and maintains the continual supply of blood and transports nutrition, oxygen, and stem cells for the regeneration of the vasculature (Saghiri et al., 2015).

Angiogenesis is the development of new blood vessels from existing blood capillaries, which is crucial for wound healing and tissue repair/regeneration (Yan et al., 2012). Angiogenesis is a multifaceted procedure that is controlled by cellular and molecular communications encompassing endothelial cell proliferation and migration, degradation of the ECM, tube creation, and development into efficient blood vessels (Mathew and Bhonde, 2017). The interchange of pro-angiogenic and anti-angiogenic signals and factors, such as growth factors, MMPs, enzymes, adhesion molecules, cytokines, and chemokines, is necessary throughout the formation of blood vessels and their development. DPSCs are very angiogenic, and they facilitate the vascularization of tissues through two distinctive mechanisms: (a) angiogenic factor or paracrine secretions by resident endothelial cells to augment vascularization or (b) direct differentiation into the vascular endothelium by imitating vasculogenesis and development (Garrido et al., 2018). Recently, several studies have reported that DPSCs secrete pro-angiogenic and anti-angiogenic proteins under diverse culturing conditions, which in turn affect the phases of angiogenesis (Markowska et al., 2018). In addition, DPSCs are adept at endorsing tube formation in HUVECs in vitro in a paracrine manner as well as with a co-culture method (Ribot et al., 2017).

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral anti-hyperglycemic medication extensively used for the treatment and management of type 2 diabetes mellitus (Pryor and Cabreiro, 2015). The primary mechanism of action is regulated by the activation of AMPK, which functions as an energy sensor by monitoring the AMP/ATP status of the cell (Patil et al., 2018). There are controversial reports on the use of metformin for angiogenesis. Metformin has been claimed to improve angiogenesis in polycystic ovary syndrome. However, numerous AMPK-independent properties of metformin have been reported, implying that metformin has pleiotropic properties (AS et al., 2018). Metformin affects the regulation of angiogenesis in in vivo and in vitro study models, and it is capable of facilitating or disrupting neo-vascularization largely by regulating the expression of VEGF (Dallaglio et al., 2014). The therapeutic manipulation of angiogenesis comprises anti-angiogenic therapies to combat tumor formation and pro-angiogenic therapies for cardiovascular and wound disorders that facilitate the formation of new blood capillaries that supply blood to impaired tissues (Han et al., 2018).

Thus, in this investigation, we aimed to determine whether metformin pre-treatment enhances the angiogenic ability of the secretome of DPSCs.

2. Materials and methods

The study protocol was approved by the Institutional Ethical Committee of Sri Venkateswara Dental College and Hospital, Chennai, India (IEC/SVDCH/2115).

2.1. In vitro culture of DPSCs

The isolation and characterization of DPSCs were carried out using the explant culture method described previously. Briefly, pulp tissue was minced into tiny fragments, and the pieces were placed in 35 mm polystyrene plastic culture dishes. A sufficient amount of fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was added to the tissues to cover them completely. The explant tissue containing was incubated at 37 °C with 5% CO_2 for 24 h; the entire culture system for the DPSCs was further maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS and antibiotic–antimycotic solution under the same temperature and CO2 conditions. The culture medium was replenished twice weekly, and cell growth, health, and morphology were monitored regularly using an inverted phase-contrast microscope. At 70–80% of confluence, the cells were dissociated using 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA) and transferred to a larger 25-cm^2 polystyrene culture flask (Nunc, Rochester, NY, USA). The confluent DPSCs were dissociated using 0.25% trypsin-EDTA solution and continuously passaged for expansion and further experiments. The cells from passages 2–4 were used in the experiments.

2.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for the proliferation of the cells

The cytotoxicities of various concentrations of metformin applied to the DPSCs were assessed using the MTT assay. The DPSCs were seeded into 96-well plates at a cell density of 1×10^4 cells per well and incubated for 24 h. For the MTT assay, four experimental groups were created: control (untreated), 5 μM metformin, 10 μM metformin, and 20 μM metformin. The cultures were incubated for 48 h at 37 °C in a 5% CO_2 incubator. Next, the MTT solution (Sigma-Aldrich Corp., St. Louis, MO, USA), at a concentration of 0.5 mg/ml, was mixed in each well after the mixing plates were incubated for 4 h at 37 °C. Subsequently, the medium was removed, and 100 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each well. The absorbance was measured at 570 nm using a Multiskan Spectrum spectrophotometer (Thermo Scientific, San Jose, CA, USA).

2.3. Real-time quantitative PCR for the analysis of gene expression

The DPSCs were seeded into 12-well plates at a cell density of 1×10^5 cells per well and incubated for 24 h. Four experimental groups were created for the gene expression analysis: control, 5 μM metformin, 10 μM metformin, and 20 μM metformin. The cultures were incubated for 48 h at 37 °C in a 5% CO_2 incubator. The total RNA was extracted from the cells using the GeneJET RNA Purification Kit (Thermo Scientific, Lithuania). RNA (2 μg) was reverse transcribed using a cDNA synthesis kit (High Capacity, Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's guidelines. A total of 100 ng of cDNA was used for a total reaction volume of 20 μg for each gene. A quantitative analysis of the genes of interest was carried out using the SYBR Green PCR master mix (Applied Biosystems, Austin, TX, USA) of a Real-Time PCR system (QS5, Applied Biosystems, Foster City, CA, USA). The expression of the target genes related to angiogenesis was normalized to that of GAPDH, as a reference gene, using the ΔΔCt

Table 1
List of primers.

Gene	Forward Primer	Reverse Primer
VEGFA	5′-TTG CCT TGC TGC TCT ACC TCC A-3′	5′-GAT GGC AGT AGC TGC GCT GAT A-3′
FGF2	5′-AGC GGC TGT ACT GCA AAA ACG G-3′	5′-CCT TTG ATA GAC ACA ACT CCT CTC-3′
CXCL8	5′-GAG AGT GAT TGA GAG TGG ACC AC-3′	5′-CAC AAC CCT CTG CAC CCA GTT T-3′
GAPDH	5′-GTC TCC TCT GAC TTC AAC AGC G-3′	5′-ACC ACC CTG TTG CTG TAG CCA A-3′

method. The list of genes and primers (Eurofins) is provided in Table 1.

2.4. DPSC-conditioned medium preparation

For the collection of the DPSC-conditioned medium (DPSC-CM), the DPSCs were seeded into 6-well plates at a seeding density of 1×10^6 cells per well with DMEM + 10% FBS. For the CM collection, four experimental groups were created: control, 5 μM metformin, 10 μM metformin, and 20 μM metformin. After 48 h of incubation, the spent medium (DPSC-CM) was aspirated and collected in sterile tubes. The DPSC-CM was stored at −80 °C until further use.

2.5. Enzyme-linked immunosorbent assay (ELISA) for the analysis of angiogenic factors in the DPSC-CM

The VEGF and angiopoietin-2 protein levels were analyzed using the KRIBIOLISA human ELISA kits (Krishgen Biosystems, Los Angeles, CA, USA). All the saliva samples were diluted 10 times in PBS, and the protocol was performed according to the experimental instructions provided with the kit. The absorbance was read at 450 nm using a spectrophotometer (Multiskan FC, Thermo Scientific, San Jose, CA, USA).

2.6. Testing of the DPSC secretome for angiogenesis employing the in vivo yolk sac membrane (YSM) model

The YSM assay was performed as previously described elsewhere (AS et al., 2018). Briefly, recently fertilized eggs of the white leghorn chicken were acquired from the hatchery and windowed at a suitable stage of embryo development. The eggs were distributed into appropriate groups. Each group consisted of three eggs. The CMs used for treatment were directly dropped onto the vasculature on the YSM of eggs. After treatment, the windows were sealed using tape. After incubation for 24 h at 37.5 °C with 70–80% of humidity. After 24 h, the shells of the eggs were cut open to the equatorial area to uncover the whole spread of blood vessels on YSM. Photographs of the YSMs were captured using a camera for comparative analysis and quantitation. Images of the YSMs were selected and resized appropriately. A square of appropriate size was gated on the parts where the effect of the CM was observed. The total number of quaternary blood vessels was manually counted. The angiogenic effects of the various CMs were compared with those of parallel controls.

2.7. Statistical analysis

Each experiment was performed in triplicate. The results are presented as the mean ± standard deviation of three independent experimental values for all the three samples. Each treatment group was individually compared with the control group, and the data for each cytokine were analyzed using unpaired *t*-test (two-tailed); GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA) was used for the analysis. $P < 0.05$ was considered as significant, and $p < 0.01$ was considered as highly significant (ns not significant, $^*p < 0.05$, and $^{**}p < 0.01$).

3. Results

3.1. Metformin does not affect the metabolic activity of DPSCs

The **DPSCs treated with** three different concentrations of metformin were compared with the control DPSCs without any treatment (Fig. 1A-1D). There was no significant increase or decrease in the metabolic activity of the DPSCs after metformin treatment, even at the high concentration of 20 μM (Fig. 1E).

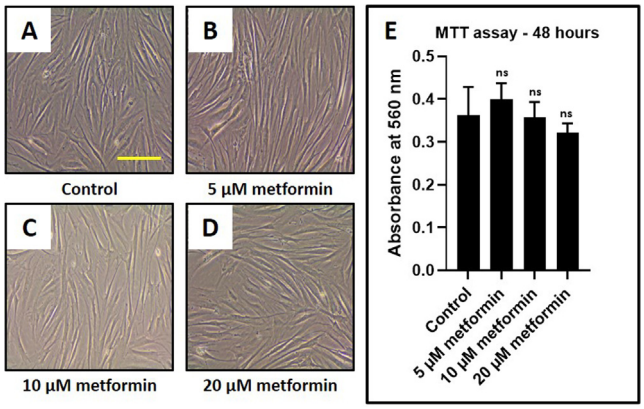

Fig. 1. Metformin treatment to DPSCs and MTT assay to check the cytotoxicity to DPSCs after treatment with different concentrations of metformin. (A-D) Appearance of the DPSCs treated with different concentrations of metformin. Scale bar = 100 μm. (E) Comparative analysis of proliferation of DPSCs was assessed MTT assay. (n = 3). ns not significant, $^*p < 0.05$, $^{**}p < 0.01$.

3.2. Metformin treatment augments the expression of angiogenesis-related genes and triggers angiogenic factor secretion in DPSCs

DPSCs treated with metformin demonstrated significantly increased expression of the VEGFA, FGF2, and CXCL8 genes (Fig. 2-A-2C, Table 2). In addition, metformin treatment significantly augmented VEGF and angiopoietin-2 secretion in the conditioned media of the DPSCs (Fig. 2D and 2E, Table 3).

3.3. Conditioned media from metformin pre-treated DPSCs significantly enhance angiogenesis in in ovo models of YSM

As evidenced in the YSM models treated with conditioned media obtained from the metformin pre-treated DPSCs, angiogenesis increased significantly in a dose-dependent manner (Fig. 3A-3H). DPSC-CM alone showed enhanced angiogenesis, comparable to that in cells treated with arginine. Angiogenesis increased with increasing concentrations of metformin (Fig. 4). Compared with treatment with controls and lower concentrations of metformin, treatment with DPSC-conditioned media pretreated with higher concentrations of metformin facilitated superior vasculature with a significantly increased number of quaternary blood vessels in comparison (Table 4).

4. Discussion

The regenerative potential of DPSCs is well-established, although its clinical application is largely impeded by senescence. Thus, researchers have constantly used biomolecules to prolong the regenerative potential of DPSCs by inhibiting senescence. Metformin has been shown to attenuate senescence in DPSCs with 100 μM metformin, inducing the highest rate of DPSC proliferation (Zhang et al., 2021). In addition to inhibiting senescence, researchers have explored ways to augment several key properties, including the potential for odontogenic, osteogenic, and angiogenic differentiation, which carry a wide array of potential clinical applications. The odontogenic potential of DPSCs was augmented by seeding into a scaffold of calcium phosphate cement containing chitosan and metformin (Qin et al., 2018). Metformin-containing resin was used to augment the regenerative potential of DPSCs by enhancing their odontogenic potential and mineral-

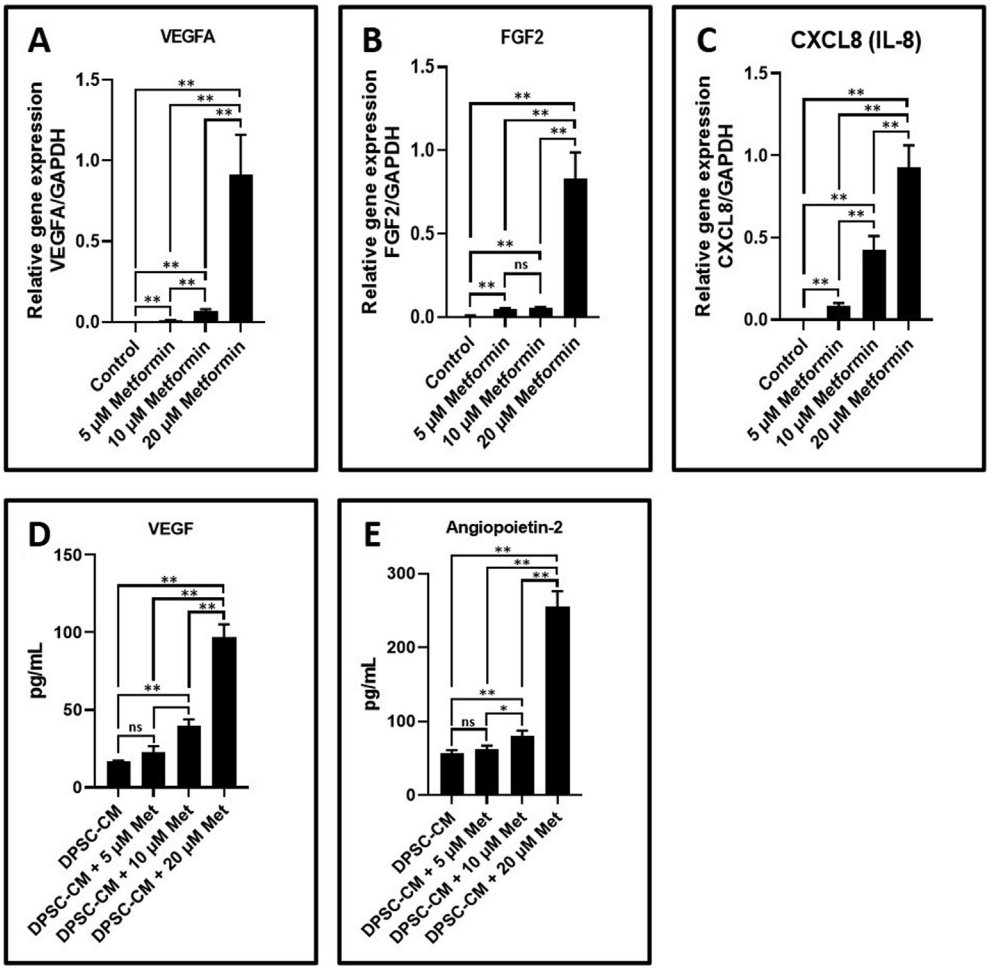

Fig. 2. Metformin treatment to DPSCs to check the gene expression of angiogenesis related genes and angiogenic factors in DPSC-CM. (A-C) Comparative gene expression analysis of VEGFA, FGF2, and CXCL8 in with and without metformin treated DPSCs. (D & E) Conditioned media from DPSCs were evaluated by using ELISA to decide the protein expression levels of VEGF and angiopoietin-2. (n = 3). ns not significant, *p < 0.05, **p < 0.01. VEGFA: Vascular endothelial growth factor A, FGF2: Basic fibroblast growth factor, CXCL8: Chemokine (C-X-C motif) ligand 8, VEGF: Vascular endothelial growth factor, DPSC-CM: Dental pulp stem cell-conditioned media, Met: Metformin.

Table 2
Relative gene expression of angiogenesis related genes.

Gene	Control	5 μM metformin	10 μM metformin	20 μM metformin
VEGFA	0.000546 ± 0.000158	0.0096 ± 0.0029	0.065 ± 0.013	0.91 ± 0.24
FGF2	0.00874 ± 0.0016	0.046 ± 0.0091	0.052 ± 0.0082	0.83 ± 0.15
CXCL8	0.00259 ± 0.00049	0.082 ± 0.019	0.42 ± 0.082	0.92 ± 0.13

Table 3
Angiogenic factors in DPSC-CM.

Angiogenic factors	DPSC-CM	DPSC-CM + 5 μM Met	DPSC-CM + 10 μM Met	DPSC-CM + 20 μM Met
VEGF	16.62 ± 0.70	22.83 ± 3.89	40.09 ± 3.83	96.75 ± 8.48
Angiopoietin-2	56.86 ± 4.26	62.23 ± 5.13	80.32 ± 7.23	255.07 ± 21.16

synthesizing ability by 9 folds (Wang et al., 2019). Metformin at a concentration of 100 mol/L augmented the DPSC potential for osteogenic differentiation, attachment, and proliferation of biphasic granules (Houshmand et al., 2018). The use of a serum-free culture medium constituting B27, heparin, and growth factors, such as VEGF-A165, augmented the DPSC potential for vasculogenesis, as evidenced by the generation of vascular tubules in 3D cultures (Luzuriaga et al., 2020). The transduction of B-cell lymphoma 2 green fluorescent protein lentiviral particles attenuates apoptosis in DPSCs, while hypoxic conditions augment their angiogenic potential (Dissanayaka et al., 2020).

Published literature has shown that therapeutic angiogenesis for dental pulp or other tissue regeneration and repair involve signaling molecules that create an angiogenic niche. The regulatory process of angiogenesis in a spatio-temporal mode is a complex interaction between diverse pro-angiogenic and anti-angiogenic factors (Pryor and Cabreiro, 2015). The role of the DPSC secretome in promoting or inhibiting the formation of blood vessels, compris-

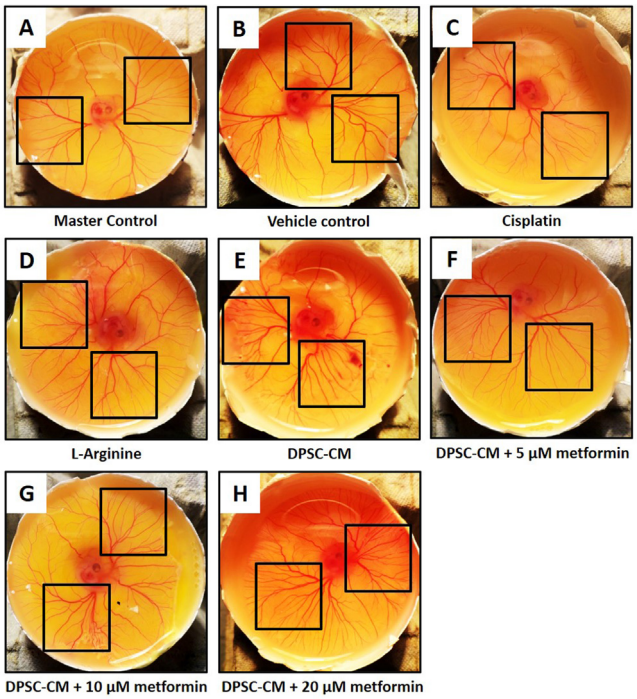

Fig. 3. Screening of angiogenesis was performed by utilizing the in ovo YSM model. (A-H) The squares around the certain areas signify the areas which were compared to quantify the angiogenesis. (n = 3).

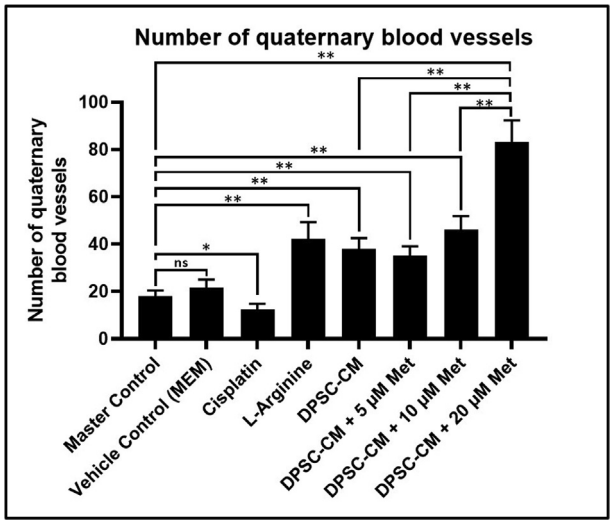

Fig. 4. Comparative effect of DPSC-conditioned medium on *in ovo* YSM blood vessels of chick embryo. Action of metformin pre-treated DPSC-conditioned medium displays better vasculature with the upsurge in the quaternary blood vessel number in comparison with control groups and lower concentrations. (n = 3). ns not significant, *p < 0.05, **p < 0.01. DPSC-CM: Dental pulp stem cell-conditioned media, Met: Metformin.

ing its impeding effects on the behavior of endothelial cells, is of great significance in optimizing the regeneration and repair of dental pulp (Mathew and Bhonde, 2017). It has been acknowledged that in vitro MSCs secrete trophic factors, including mitogenic, angiogenic, anti-apoptotic, chemoattractants, immunomodulatory, cytokines, extracellular matrix proteins, and immunomodulatory factors (Dallaglio et al., 2014). However, the secreted signaling molecules vary with cell type, duration, and conditions for culturing. Consistent with the findings of the aforementioned studies, our investigation revealed that the DPSC secretome promotes angiogenesis (Garrido et al., 2018). Remarkably, with the increased concentrations, the metformin-treated DPSC secretome showed an increasing capability to promote angiogenesis (Figs. 3 and 4). With the increasing concentrations of metformin, DPSCs showed an increase in the expression of angiogenesis-related genes and secreted angiogenic factors, which are responsible for promoting and enhancing angiogenesis (Fig. 2). This in vivo assessment established that the secretome from DPSCs can stimulate noticeable angiogenic effects. Therefore, the significant effect of the DPSC secretome on angiogenesis, otherwise with metformin, may be a consequence of the additional or synergistic exploitation of various soluble growth factors. The synergistic actions of molecules present in the secretome are important for the therapeutic claims associated with angiogenesis. Nevertheless, comprehensive proteomic examinations to determine the amount of recognized proteins responsible for angiogenesis in the DPSC secretome and in vitro and in vivo investigations are necessary to confirm the findings of this study and estimate the latent effect of metformin on the augmentation of these factors and the restoration of the tissues.

Therapeutic angiogenesis is of utmost importance in regeneration and wound healing. Stem cells are considered to have paracrine pro-angiogenic effects. Additionally, stem cells can be primed to enhance their angiogenic effects. Although we showed the enhanced angiogenic potential of DPSCs treated with metformin, it is necessary to explore the interactions between DPSCs and endothelial cells. Additionally, whether DPSCs can act as endothelial cells giving rise to blood capillaries should be investigated. Importantly, it would be interesting to determine whether DPSCs treated with metformin can induce angiogenesis in vivo.

Table 4
Number of quaternary blood vessels after treatment with DPSC-CM.

Concentration	Number of quaternary blood vessels
Master Control	18.0 ± 2.4
Vehicle Control (MEM)	21.7 ± 3.4
Cisplatin (Negative control)	12.3 ± 2.5
L-Arginine (Positive control)	42.3 ± 6.9
DPSC-CM	38.0 ± 4.5
DPSC-CM + 5 µM Met	35.3 ± 3.7
DPSC-CM + 10 µM Met	46.0 ± 5.9
DPSC-CM + 20 µM Met	83.3 ± 9.0

5. Conclusion

Pre-treatment with metformin enhanced the angiogenic potential of the secretome of DPSCs, as evidenced by the increase in the quaternary blood vessels in the chick embryo YSM model after treatment with the DPSC secretome.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

AS, M.N., Deshpande, R., Kale, V.P., Bhonde, R.R., Datar, S.P., 2018. Establishment of an in ovo chick embryo yolk sac membrane (YSM) assay for pilot screening of potential angiogenic and anti-angiogenic agents. Cell Biol. Int. 42 (11), 1474–1483. https://doi.org/10.1002/cbin.v42.1110.1002/cbin.11051.

Bakhashab, S., Ahmed, F.W., Schulten, H.-J., Bashir, A., Karim, S., Al-Malki, A.L., Gari, M.A., Abuzenadah, A.M., Chaudhary, A.G., Alqahtani, M.H., Lary, S., Ahmed, F., Weaver, J.U., 2016. Metformin improves the angiogenic potential of human CD34+ cells co-incident with downregulating CXCL10 and TIMP1 gene expression and increasing VEGFA under hyperglycemia and hypoxia within a therapeutic window for myocardial infarction. Cardiovasc. Diabetol. 15, 27. https://doi.org/10.1186/s12933-016-0344-2.

Bhandi, S., Al Kahtani, A., Mashyakhy, M., Alsofi, L., Maganur, P.C., Vishwanathaiah, S., Testarelli, L., Del Giudice, A., Mehta, D., Vyas, N., Patil, V.R., Raj, A.T., Patil, S., 2021. Modulation of the Dental Pulp Stem Cell Secretory Profile by Hypoxia Induction Using Cobalt Chloride. J. Pers. Med. 11, 247. https://doi.org/10.3390/jpm11040247.

Dallaglio, K., Bruno, A., Cantelmo, A.R., Esposito, A.I., Ruggiero, L., Orecchioni, S., Calleri, A., Bertolini, F., Pfeffer, U., Noonan, D.M., Albini, A., 2014. Paradoxic effects of metformin on endothelial cells and angiogenesis. Carcinogenesis 35, 1055–1066. https://doi.org/10.1093/carcin/bgu001.

Di Pietro, M., Parborell, F., Irusta, G., Pascuali, N., Bas, D., Bianchi, M.S., Tesone, M., Abramovich, D., 2015. Metformin Regulates Ovarian Angiogenesis and Follicular Development in a Female Polycystic Ovary Syndrome Rat Model. Endocrinology 156 (4), 1453–1463. https://doi.org/10.1210/en.2014-1765.

Dissanayaka, W.L., Han, Y., Zhang, L., Zou, T., Zhang, C., 2020. Bcl-2 Overexpression and Hypoxia Synergistically Enhance Angiogenic Properties of Dental Pulp Stem Cells. Int. J. Mol. Sci. 21, 6159. https://doi.org/10.3390/ijms21176159.

Ducret, M., Fabre, H., Degoul, O., Atzeni, G., McGuckin, C., Forraz, N., Alliot-Licht, B., Mallein-Gerin, F., Perrier-Groult, E., Farges, J.-C., 2015. Manufacturing of dental pulp cell-based products from human third molars: current strategies and future investigations. Front. Physiol. 6. https://doi.org/10.3389/fphys.2015.00213.

García-Bernal, D., López-García, S., Sanz, J.L., Guerrero-Gironés, J., García-Navarro, E. M., Moraleda, J.M., Forner, L., Rodríguez-Lozano, F.J., 2021. Melatonin Treatment Alters Biological and Immunomodulatory Properties of Human Dental Pulp Mesenchymal Stem Cells via Augmented Transforming Growth Factor Beta Secretion. J. Endod. 47 (3), 424–435. https://doi.org/10.1016/j.joen.2020.12.008.

Garrido, M.P., Vera, C., Vega, M., Quest, A.F.G., Romero, C., 2018. Metformin prevents nerve growth factor-dependent proliferative and proangiogenic effects in epithelial ovarian cancer cells and endothelial cells. Ther. Adv. Med. Oncol. 10, 175883591877098. https://doi.org/10.1177/1758835918770984

Gharaei, M.A., Xue, Y., Mustafa, K., Lie, S.A., Fristad, I., 2018. Human dental pulp stromal cell conditioned medium alters endothelial cell behavior. Stem Cell Res. Ther. 9, 69. https://doi.org/10.1186/s13287-018-0815-3.

Han, J., Li, Y., Liu, X., Zhou, T., Sun, H., Edwards, P., Gao, H., Yu, F.-S., Qiao, X., Chen, J., 2018. Metformin suppresses retinal angiogenesis and inflammation in vitro and in vivo. PLoS One 13 (3), e0193031. https://doi.org/10.1371/journal.pone.0193031.

Houshmand, B., Tabibzadeh, Z., Motamedian, S.R., Kouhestani, F., 2018. Effect of metformin on dental pulp stem cells attachment, proliferation and differentiation cultured on biphasic bone substitutes. Arch. Oral Biol. 95, 44–50. https://doi.org/10.1016/j.archoralbio.2018.07.012.

Kim, H., Han, J.W., Lee, J.Y., Choi, Y.J., Sohn, Y.-D., Song, M., Yoon, Y.-S., 2015. Diabetic Mesenchymal Stem Cells Are Ineffective for Improving Limb Ischemia Due to Their Impaired Angiogenic Capability. Cell Transplant. 24 (8), 1571–1584. https://doi.org/10.3727/096368914X682792.

Luzuriaga, J., Irurzun, J., Irastorza, I., Unda, F., Ibarretxe, G., Pineda, J.R., 2020. Vasculogenesis from Human Dental Pulp Stem Cells Grown in Matrigel with Fully Defined Serum-Free Culture Media. Biomedicines 8, 483. https://doi.org/10.3390/biomedicines8110483.

Markowska, A., Sajdak, S., Huczyński, A., Rehlis, S., Markowska, J., 2018. Ovarian cancer stem cells: A target for oncological therapy. Adv. Clin. Exp. Med. 27, 1017–1020. https://doi.org/10.17219/acem/73999.

Mathew, S.A., Bhonde, R., 2017. Mesenchymal stromal cells isolated from gestationally diabetic human placenta exhibit insulin resistance, decreased clonogenicity and angiogenesis. Placenta 59, 1–8. https://doi.org/10.1016/j.placenta.2017.09.002.

Patil, V.R., Kharat, A.H., Kulkarni, D.G., Kheur, S.M., Bhonde, R.R., 2018. Long term explant culture for harvesting homogeneous population of human dental pulp stem cells. Cell Biol. Int. 42 (12), 1602–1610. https://doi.org/10.1002/cbin.v42.1210.1002/cbin.11065.

Pryor, R., Cabreiro, F., 2015. Repurposing metformin: an old drug with new tricks in its binding pockets. Biochem. J. 471, 307–322. https://doi.org/10.1042/BJ20150497.

Qin, W., Chen, J.-Y., Guo, J., Ma, T., Weir, M.D., Guo, D., Shu, Y., Lin, Z.-M., Schneider, A., Xu, H.H.K., 2018. Novel Calcium Phosphate Cement with Metformin-Loaded Chitosan for Odontogenic Differentiation of Human Dental Pulp Cells. Stem Cells Int. 2018, 1–10. https://doi.org/10.1155/2018/7173481.

Raj, A.T., Kheur, S., Bhonde, R., Gupta, A.A., Patil, S., 2021. Assessing the effect of human mesenchymal stem cell-derived conditioned media on human cancer cell lines: A systematic review. Tissue Cell 71, 101505. https://doi.org/10.1016/j.tice:2021.101505.

Rena, G., Hardie, D.G., Pearson, E.R., 2017. The mechanisms of action of metformin. Diabetologia 60 (9), 1577–1585. https://doi.org/10.1007/s00125-017-4342-z.

Ribot, J., Caliaperoumal, G., Paquet, J., Boisson-vidal, C., Petite, H., Anagnostou, F., 2017. Type 2 diabetes alters mesenchymal stem cell secretome composition and angiogenic properties. J. Cell Mol. Med. 21 (2), 349–363. https://doi.org/10.1111/jcmm.2017.21.issue-210.1111/jcmm.12969.

Rodríguez-Lozano, F., Insausti, C., Iniesta, F., Blanquer, M., Ramírez, M., Meseguer, L., Meseguer-Henarejos, A., Marín, N., Martínez, S., Moraleda, J., 2012. Mesenchymal dental stem cells in regenerative dentistry. Med. Oral Patol. Oral y Cir. Bucal, e1062–e1067. https://doi.org/10.4317/medoral.17925.

Rodríguez-Lozano, F.J., Moraleda, J.M., 2011. Use of dental stem cells in regenerative dentistry: A possible alternative. Transl. Res. 158 (6), 385–386. https://doi.org/10.1016/j.trsl.2011.07.008.

Saghiri, M.A., Asatourian, A., Sorenson, C.M., Sheibani, N., 2015. Role of Angiogenesis in Endodontics: Contributions of Stem Cells and Proangiogenic and Antiangiogenic Factors to Dental Pulp Regeneration. J. Endod. 41 (6), 797–803. https://doi.org/10.1016/j.joen.2014.12.019.

Wang, S., Xia, Y., Ma, T., Weir, M.D., Ren, K.e., Reynolds, M.A., Shu, Y., Cheng, L., Schneider, A., Xu, H.H.K., 2019. Novel metformin-containing resin promotes odontogenic differentiation and mineral synthesis of dental pulp stem cells. Drug Deliv. Transl. Res. 9 (1), 85–96. https://doi.org/10.1007/s13346-018-00600-3.

Yan, J., Tie, G., Wang, S., Messina, K.E., DiDato, S., Guo, S., Messina, L.M., 2012. Type 2 Diabetes Restricts Multipotency of Mesenchymal Stem Cells and Impairs Their Capacity to Augment Postischemic Neovascularization in db/db Mice. J. Am. Heart Assoc. 1 (6). https://doi.org/10.1161/JAHA.112.002238.

Zhang, S., Zhang, R., Qiao, P., Ma, X., Lu, R., Wang, F., Li, C., E, L., Liu, H., Facchin, F., 2021. Metformin-Induced MicroRNA-34a-3p Downregulation Alleviates Senescence in Human Dental Pulp Stem Cells by Targeting CAB39 through the AMPK/mTOR Signaling Pathway. Stem Cells Int. 2021, 1–13. https://doi.org/10.1155/2021/6616240.