Stem Cell Secretome Modulated by Arsenicum album 30C Ameliorates Lipopolysaccharide-induced Cytokine Storm in Blood Mononuclear Cells in vitro

Parth Aphale1,* Avinash Sanap2,* Dharmendra Sharma1 Avinash Kharat2 Supriya Kheur2 Chinmay Gawade1 Indumati Somasundaram3 Ramesh Bhonde2

1 Dr D. Y. Patil Homeopathic Medical College and Research Centre, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India
2 Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India
3 Department of Biotechnology Engineering, Kolhapur Institute of Technology’s College of Engineering, Kolhapur, India

Address for correspondence Ramesh Bhonde, PhD, Professor Emeritus, Dr. D. Y. Patil Vidyapeeth, Sant Tukaram Nagar, Pimpri-411 018, Pune (Maharashtra), India (e-mail: rrbhonde@gmail.com).

Homeopathy 2024;113:132–141.

Abstract

Background The therapeutic effectiveness of mesenchymal stem cells (MSCs) and their secretome can be enhanced by means of physical, chemical and biological preconditioning. Arsenicum album 30C (AA30) has been one of the leading homeopathic medicines used in prophylaxis against SARS-CoV-2 infection.

Aims This study aimed to investigate whether AA30 preconditioning could influence the growth factors and cytokine profile of the human dental pulp-derived MSC (DPD-MSC) secretome. Also, to test the efficacy of the AA30-preconditioned DPD-MSC secretome in ameliorating the lipopolysaccharide (LPS)-induced cytokine storm in human peripheral blood mononuclear cells (PBMCs) as an in-vitro cellular model.

Methods The cytotoxicity of AA30 was assessed in DPD-MSCs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Growth factors and cytokine levels in the AA30-preconditioned DPD-MSC secretome were analysed by fluorescence-activated cell sorting (FACS) analysis. The angiogenic potential of the AA30-preconditioned DPD-MSC secretome was assessed by chick yolk-sac membrane (YSM) assay. Culture medium with 0.001% ethanol was used as vehicle control. The efficacy of the AA30-preconditioned DPD-MSC secretome in ameliorating the cytokine storm was assessed in LPS pre-treated PBMCs. The mRNA and protein expression of inflammatory markers such as IL-1β, IL-6 and IL-10 were analysed by using RT-PCR and FACS analysis respectively.

Results AA30 did not exhibit cytotoxicity in the concentration range of 1% to 50%. Furthermore, the AA30-preconditioned DPD-MSC secretome exhibited a significant increase in the levels of angiogenic factors, such as human angiopoietin-2, EPO and PDGF-AA, and decreased levels of cytokines, such as TNF-α, CXCL-8 and IL-6. The AA30-preconditioned DPD-MSC secretome showed augmented angiogenesis compared to vehicle controls. The DPD-MSC secretome ameliorated LPS-induced mRNA and protein expression of IL-1β, IL-6 and IL-10 in PBMCs.

Keywords
► Arsenicum album 30C
► COVID-19
► Cytokine storm
► Inflammation
► Mesenchymal Stem Cells

* Equal first authors.
Conclusion The AA30-preconditioned DP-D-MSC secretome augmented angiogenesis and ameliorated the LPS-induced cytokine storm in human PBMCs \textit{in vitro}. Our data demonstrate that AA30 preconditioning enhances the therapeutic potency of MSCs and their secretome.

Introduction

Coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 infection was declared a global pandemic by the World Health Organization (WHO) in March 2020.\(^1\) SARS-CoV-2 infection is known to cause overactivation of the immune system, which results in a burst of cytokine release alternatively known as a ‘cytokine storm’\(^2\). SARS-CoV-2-mediated cytokine storms are known to cause alveolar injury and hyaline membrane formation, which eventually lead to multiple organ failure.\(^3\) Among proinflammatory cytokines, the release of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1\(\beta\) (IL-1\(\beta\)) followed by interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)) and MIP-1\(\beta\) are primary drivers of alveolar injury.\(^4\)

Various treatment modalities have been explored for the clinical management of the disease.\(^5\) Globally, traditional medicines have been in clinical use for centuries. Herbal and homeopathic medicines are actively used in the absence of effective allopathic therapies.\(^6\) Administration of the homeopathic ‘genus epidemicus’ as a prophylactic homeopathic treatment in symptomatic cases is often considered an inexpensive, safe and feasible approach.\(^7\) The preventive aspect of homeopathy is well known, including for viral infections. Homeopathy has stood the test of time over two centuries as a notable approach in controlling morbidity and epidemics. Historically, homeopathy gained prominence following its usefulness for the management of epemics such as cholera, typhoid, yellow fever, diphtheria, Spanish flu, and scarlet fever.\(^8\) In India, Asernicum album 30C (AA30) was one of the most frequently prescribed homeopathic prophylactic medicines during the COVID-19 pandemic as per the health advisory given by the Ministry of AYUSH, Government of India, against coronavirus infection.\(^9\) However, the molecular and cellular mechanisms of action of AA30 in the prevention and management of mild-to-moderate cases of COVID-19 remain largely unknown.

Mesenchymal stem cells (MSCs) are plastic, adherent and spindle-shaped, and have multi-lineage differentiation potential.\(^10\) MSCs possess potent immunomodulatory functions, which can be attributed to the paracrine activity of secreted growth factors, cytokines, small bioactive proteins, micro-RNAs and extracellular vesicles, including exosomes, collectively known as the ‘secretome’\(^11\). MSCs have been proven to be effective in a wide range of systemic and tissue-specific disorders.\(^12\) MSCs and their secretome are known to be angiogenic in nature, which fosters regeneration.\(^13\) The therapeutic potency of the MSC secretome can be enhanced by employing the approach of ‘preconditioning’, which involves modulation of culture conditions (hypoxia, 3D cell culture, etc.), pharmaceutical and herbal drug treatment in the \textit{in-vitro} culture of cells.\(^14\) Preconditioning of MSCs by means of physical, chemical and biological factors without affecting stemness is an effective approach to improve stem cell functionality in the clinical management of disease.\(^15\) Additionally, prior exposure of MSCs to a disease microenvironment potentiates their therapeutic function, providing better adaptation of tissues for post-transplantation survival.\(^16\) Studies have proven that the MSC secretome can be enriched with desired bioactive factors with preconditioning and tailored for the specific requirements of the disease. For instance, preconditioning with traditionally used herbal medicine has been shown to increase the proliferation and differentiation of human MSCs into various lineages.\(^17\) Culture condition modification such as hypoxia is known to enhance the survival and angiogenic potential of human MSCs.\(^18\) MSCs can thus be guided towards the desired functions and cellular fate by providing an appropriate microenvironment or ‘niche’.\(^19\)

Importantly, in the context of the present study, ascorbic acid and IFN\(\gamma\) preconditioning of human MSCs have been shown to improve the potency of the MSC secretome in ameliorating the lipopolysaccharide (LPS)-induced cytokine storm in human peripheral blood mononuclear cells (PBMCs) \textit{in vitro}.\(^20\)

During the COVID-19 pandemic, MSCs and their secretome were actively investigated for the management of COVID-19 owing to their immunomodulatory and pro-angiogenic activity.\(^21,22\) Though the safety of MSCs for human interventions is well established, maintaining uniform efficacy remains a challenging task due to factors such as donor-associated variations, batch-to-batch variations during \textit{in-vitro} expansion and limited growth potential.\(^23\) Therefore, a strategic program for preconditioning cells is an attractive option to improve the therapeutic efficacy of stem cells and secretome.

Dental pulp obtained from extracted teeth is an ideal source of MSCs due to the ease of isolation and minimal ethical concerns, making it an ideal candidate for cell culture studies.\(^24\) The current study aimed to investigate the effects of AA30 preconditioning on the human dental pulp-derived mesenchymal stem cell (DP-D-MSC) secretome: cytotoxicity, cell proliferation, growth factor and cytokine profile, and angiogenic potential. Since LPS is known to induce a pro-inflammatory response \textit{in vitro} in PBMCs, similar to the effect of SARS-CoV-2 spike protein,\(^25\) the potential of the AA30-preconditioned DP-D-MSC secretome to ameliorate the LPS-induced cytokine storm in PBMCs was then investigated as a further \textit{in-vitro} cellular model.
Materials and Methods

A pictorial description of the entire experiment's methods is shown in – Supplementary File 1 (available in the online version of the paper).

Isolation of DPD-MSCs

All study protocols for the isolation and characterization of DPD-MSCs were approved by the Institutional Committee of Stem Cell Research (IC-SCR) of Dr. D. Y. Patil Vidyapeeth, Pune, India (No. IC-SCR/RM/40/21). Institutional guidelines and regulations were followed in the protocols involving human samples. Informed consent was obtained from the patients prior to sample collection. DPD-MSCs were isolated by the explant culture method using previously published protocols. Pre-molar teeth were obtained from healthy individuals aged 15-30 years undergoing orthodontic treatment (n = 7). Pulp was removed from the teeth under aseptic conditions and washed with phosphate buffer saline (PBS, pH 7.4) containing 1% antibiotic–antimycotic solution (Gibco). The pulp was cut into small pieces and placed into 12-well cell culture plates. A drop of serum was placed on top of the explant and incubated at 37°C in a humidified CO2 incubator. The next day, complete culture medium (alpha-minimal essential medium [α-MEM] along with 10% fetal bovine serum [FBS] and 1% antibiotic–antimycotic solution) was added to the explant, and cell growth was monitored for the next 7 to 8 days under a microscope. Cells were detached from explants by trypsinization and sub-cultured in fresh T25 cell culture flasks.

Characterization of DPD-MSCs

Surface Marker Expression

Cells were isolated, and passage 4 cells were characterized for the expression of CD90, CD73, CD105 (PE tagged), CD45, CD34 and HLA-DR (FITC tagged) (BD Biosciences) by flow cytometry as previously described by us.27 Tri-lineage Differentiation

Passage 4 cells were seeded in 24-well cell culture plates until they achieved 80% to 90% confluence. Cells were further differentiated into osteocytes (Stempro, A1007201), adipocytes (Stempro, A1007001) and chondrocytes (Stempro, A1007101), using a standard cocktail for 18, 15 and 21 days respectively. Once the cells were differentiated, they were stained with Alizarin red, oil red O and Alcian blue, followed by microscopic examination under a light microscope.

Assessment of Cell Cytotoxicity/Proliferation

Arsenicum album with a potency of 30C (AA30) was used in a liquid form for all experiments. AA30 was obtained from Dr. Willmar Schwabe India Pvt. The cytotoxicity of AA30 was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously.28 DPD-MSCs at a density of 1 × 104 cells per well were seeded in 96 well plates. Once the cells adhered, they were incubated with different concentrations (1, 5, 10, 20, 40 and 50%) of AA30 diluted in culture medium for 48 hours. Culture medium containing 0.001% ethanol was kept as a vehicle control. After incubation, the proliferation rate was determined by MTT assay. Briefly, 50 μl of MTT (5 mg/ml) was added to each well and incubated at 37°C for 4h. Then, the MTT solution was removed, and the insoluble blue formazan crystals were dissolved in 100 μl of dimethyl sulfoxide. The absorbance was measured at 570 nm using a microplate reader.

Preconditioning of DPD-MSCs with AA30

DPD-MSCs were seeded in 35 mm cell culture plates at a density of 1 × 104 cells per well. Once cells were 80-85% confluent, fresh α-MEM medium (without serum) was added along with 1% AA30 (diluted in culture medium). Cells were incubated for 48 hours at 37°C and 5% CO2. Culture medium containing 0.001% ethanol AA30 was kept as vehicle control.

Collection of AA30-preconditioned DPD-MSC Secretome

DPD-MSCs were treated with 1% AA30 as described above. After the 48 h incubation, the culture medium was collected and filtered through 0.22 μm filters. The DPD-MSC secretome was stored at ~80°C until further use. A 50% DPD-MSC secretome diluted with α-MEM (without serum) was used for all experiments.

Growth Factor and Cytokine Analysis of the DPD-MSC Secretome

Growth factor and cytokine analysis in the secretome pre-treated with AA30 was performed by using the MACS Plex cytokine assay kit (Miltenyi Biotec Inc., CA, USA) and growth factor assay kit (LEGENDplex Multianalyte flow assay kit 13-plex; Biolegend, San Diego, CA, USA) on a FACS analyser. The secretome obtained from DPD-MSCs was subjected to human cytokine (GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and TNF-α) and growth factor (Ang-2, EGF, erythropoietin [EPO], FGF-b, G-CSF, GM-CSF, HGF, M-CSF, platelet-derived growth factor (PDGF)-AA, PDGF-BB, SCF, TGF-α and VEGF) analysis according to the manufacturer’s instructions. A culture medium containing 0.001% ethanol AA30 was used as a vehicle control to compare the levels of growth factor and cytokine levels.

In-ovo Yolk-sac Membrane Assay for Assessment of Angiogenic Potential of the DPD-MSC Secretome

Animal ethics approval was obtained from the Institutional Animal Ethics Committee of Dr. D. Y. Patil Medical College and Research Center, Pune (India; IAEC No. CPCSEA/DYPMC/IAEC/05/2019). The yolk-sac membrane (YSM) assay was employed to assess the angiogenic potential of the DPD-MSC secretome preconditioned with AA30, as previously described.29 The YSM assay minimizes the animal experimentation of inoculating animals subcutaneously for testing angiogenic agents. It reduces animal suffering and reduces or even replaces whole-animal experiments, supporting the 3Rs principle of reduction, replacement and refinement.
The use of human stem cells and mononuclear cells in determining the action of AA30 also supports the 3Rs principle, discouraging the use of animals in research. The use of human stem cells as a screening platform for new drugs is far better than performing animal experiments. It is easy and economical in terms of cost and time. Previously, we proposed a human stem cell platform for screening ayurvedic and homeopathic medicines as an alternative to animal use in research, reinforcing the 3Rs principle.30

Freshly laid eggs were procured from Venkateshwar Hatcheries Pvt. Ltd., Pune, India and subjected to 48 h incubation in a humidified incubator at 37°C. After incubation, a small hole was made using forceps on the blunt side of the eggs, and 45 ml of albumin was removed. Furthermore, 100 µL of DPD-MSC secretome and AA30-preconditioned DPM-MSC secretome was added on top of the YSM. Culture medium without preconditioning of AA30 was kept as the vehicle control. Eggs were sealed and incubated for 48 hours. Blood vessel formation was captured using a camera, and quantitative analysis of vessel density, total vessel network length and total segments was performed using the Wim-CAM online tool, as previously described.

Cytokine Analysis in PBMC Culture Medium
The culture supernatant was subjected to cytokine analysis by using the MACS Plex Cytokine Analysis Kit (Miltenyi Biotec Inc., CA, United States), as above. The culture supernatant was briefly mixed with MACS Plex Cytokine capture beads followed by incubation for 2 hours in the dark. Upon centrifugation, pellets were washed and incubated with MACS Plex Cytokine 12 detection reagent for an hour. Pellets obtained upon centrifugation were suspended in 200 µL of MACS Plex buffer and acquired on an NxT acoustic focusing cytometer (Thermo Fisher). The results obtained on the cytometer were converted into pg/mL after comparing the readings with standards.

Gene Expression Studies in Peripheral Blood Mononuclear Cell Culture Medium
The mRNA expression of key cytokines, such as IL-1β and IL-6, was analysed by using RT-PCR. TRIzol (Gibco, United States) was employed to isolate total RNA from PBMCs, which was reverse transcribed into cDNA by a high-capacity reverse transcription kit (Applied Biosystems, United States). Then, the mRNA expression of IL1β and IL-6 was analysed on Quant Studio 5 (Applied Biosystems, United States). Primer sequences and PCR conditions are described in Table 1. (denaturation, 94°C for 15 seconds; annealing and extension, 94°C for 45 seconds; followed by melting curve analysis). Transcript expression was normalized to GAPDH expression, followed by fold expression analysis by using the ΔΔCt method.

Statistical Analysis
SPSS software (version 20) was used for the statistical analysis. One-way ANOVA was followed by Tukey’s multiple comparison test to evaluate the differences between groups. Values are expressed as mean ± standard deviation (SD), and p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) were considered statistically significant.

Results
DPD-MSC Characterization
The isolated DPD-MSCs demonstrated a fibroblast-like appearance with positive expression of CD73, CD105 and CD90 and negative expression of CD45, CD34 and HLA-DR, as shown in Fig. 1a. Furthermore, DPD-MSCs differentiated into osteocytes, adipocytes and chondrocytes, as evidenced by staining with Alizarin red, oil red O and Alcian blue respectively (Fig. 1b).

Table 1 Primer sequences used in the RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCCACTCTCCACACCTTGAC</td>
<td>CTCTCTCTTTGTGCTCTTGCT</td>
<td>60</td>
<td>286</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCAGAAGCCACAGAGAAGA</td>
<td>TTGTGCTGCACTTGCTGCT</td>
<td>60</td>
<td>181</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CAAGCCGCTCTCCTGGATTCT</td>
<td>TGAACGCGGCTGATGAGG</td>
<td>60</td>
<td>233</td>
</tr>
</tbody>
</table>

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.
Cytotoxicity/proliferation of *Arsenicum album* 30C-treated DPD-MSCs

*Arsenicum album* 30C did not exert cytotoxic effects on DPD-MSCs in the concentration range of 1 to 50%, as revealed by the MTT assay (Fig. 2). We observed an apparent increase in cell proliferation at concentrations 10 and 30%, but the change was not statistically significant (p > 0.05).

*Arsenicum album* 30C Modulates the Growth Factor and Cytokine Profile of the DPD-MSC Secretome

We analysed the growth factor levels in the DPD-MSC secretome preconditioned with AA30. The analysis revealed a significant increase in the levels of human angiopoietin-2 (p = 0.029), EPO (p = 0.010) and PDGF-AA (p = 0.001), but a decrease in the levels of G-CSF (p = 0.001), HGF (p = 0.003) and M-CSF (p = 0.010). AA30 did not affect the levels of SCF significantly (Fig. 3a).

Cytokine profiling of AA30 cells preconditioned with DPD-MSCs revealed a significant decrease in TNF-α (p = 0.032), C-X-C motif chemokine ligands 8 (CXCL-8; p = 0.001) and IL-6 (p = 0.002) levels, whereas TGF-β levels (p = 0.005) were increased (Fig. 3b).

*Arsenicum album* 30C-preconditioned DPD-MSC Secretome Exhibits Increased *in-ovo* Angiogenic Potential

A yolk-sac membrane assay was performed to inspect whether AA30 preconditioning modulates angiogenesis. We observed a significant increase in blood vessel formation in the DPD-MSC secretome and AA30-preconditioned DPD-MSC secretome-treated chicken embryos, as depicted in Fig. 4a. Quantitative assessment of the blood vessels revealed a significant increase in the blood vessel density (%) (p = 0.024) and total vessel network length (p = 0.021) (Fig. 4b).

*Arsenicum album* 30C Ameliorates LPS-induced Cytokine Storm in PBMCs

The DPD-MSC secretome preconditioned with AA30 significantly down-regulated the protein expression of IL-10 (p = 0.001), IL-6 (p = 0.000) and IL-β (p = 0.019) (Fig. 5a). Additionally, RT-PCR analysis revealed a significant decrease in the expression of IL-6 (p = 0.003) and IL-1β (p = 0.007) (Fig. 5b).

Discussion

During the COVID-19 pandemic, complementary and alternative medicines were given major emphasis in clinical
management. In India, AA30 was advocated as ‘an immune booster’ as one of the government’s preventive policy measures. After the onset of the COVID-19 pandemic, AA30 was implemented in COVID-19 hot spots in India to assess its effectiveness in a multi-centre randomized controlled trial. The results of the trial showed a decrease in incidence and an increased protection against COVID-19 infection in the AA30 intervention group compared to the control.

In the current study, we investigated the effect of AA30 preconditioning on the DPD-MSC secretome, which is currently under investigation for the clinical management of SARS-CoV-2 associated alveolar damage. At the outset, we assessed the cytotoxicity of AA30 in human DPD-MSCs in the concentration range of 1 to 50% and found no significant decrease in cell viability, indicating its safe nature for human use. Our results are in agreement with previously published in-vivo reports where Arsenicum album was administered for up to 2 years without any significant toxic effects. Additionally, AA30 has been shown to decrease levels of biomarkers of arsenic toxicity in mice. In an in-vitro study, AA30 was shown to ameliorate arsenate cytotoxicity in yeast (Saccharomyces cerevisiae) by decreasing lipid peroxidation, protein carbonylation, DNA damage and ROS formation. Although multiple studies have established the safety of AA30 for human intervention, a case report published by Theruvath et al implicated AA30-associated liver injury in human subjects.

Furthermore, we observed a significant immunomodulatory effect of AA30 on the DPD-MSC secretome. Angiogenesis-promoting growth factors such as angiopoietin, EPO and PDGF-AA were significantly increased, whereas the levels of G-CSF, HGF and M-CSF were significantly decreased upon AA30 preconditioning. Additionally, the levels of pro-inflammatory cytokines, such as TNF-α, CXCL-8 and IL-6, were down-regulated, and TGF-β levels were unregulated in the DPD-MSC secretome. We were curious to know whether...
increased levels of angiopoietin, EPO and PDGF-AA could functionally translate into augmented angiogenesis. As a result, a yolk-sac membrane assay was performed, which revealed increased angiogenesis in the AA30-preconditioned DPD-MSC secretome. SARS-CoV-2 infection is known to mediate alveolar damage and disturb endothelial homeostasis.\textsuperscript{3,4,40} Therefore, AA30-mediated augmented angiogenesis might be beneficial in alveolar repair during severe COVID-19 cases.

SARS-CoV-2 entry and replication inside the host are known to over-activate the immune system, which results in the release of pro-inflammatory cytokines (cytokine storm).\textsuperscript{2} Excessive release of pro-inflammatory cytokines is one of the key hallmarks of SARS-CoV-2 mediated alveolar

**Fig. 4** (a) Yolk-sac membrane assay in chicken embryos treated with control, DPD-MSC-secretome and AA30-preconditioned DPD-MSC secretome. (b) Quantitative assessment of angiogenesis by the Wim Tube (online tool) in terms of vessel density (%) and total vessel network length. The data shown are the mean ± SD. *p < 0.05.
damage. Human SARS-CoV-2 (COVID-19) infection is characterized by a high mortality rate because some patients develop a large innate immune response associated with a cytokine storm and acute respiratory distress syndrome (ARDS). At the preliminary stage, a cytokine storm may be necessary to control the infection, though undue and maladjusted immune responses result in membrane formation, diffuse alveolar injury and fibrin exudation lung injury. Cytokine storm is characterized by the release of TNF-α and IL-1β followed by the release of anti-inflammatory mediators such as IL-10 to maintain homeostasis in the inflammatory environment. The malfunction in establishing homeostasis leads to the release of pro-inflammatory cytokines, including IL-2, IL-6, IL-8, IL-12, MIP-1α and MIP-1β.

Our results show that the DPD-MSC secretome preconditioned with AA30 possesses significantly higher potential in ameliorating the LPS-induced cytokine storm. From our results, it is evident that the DPD-MSC secretome preconditioned with AA30 effectively down-regulates the LPS-induced levels of IL-6 and IL-1β, which are linked with cytokine-associated alveolar damage and poor outcomes in hospitalized patients. It is noteworthy that drugs that block the IL-6 receptor, such as tocilizumab and sarilumab, have been considered in severe COVID-19 patients for clinical management. Conventionally, IL-6 is considered a pro-inflammatory cytokine and is associated with poor outcomes in COVID-19-affected patients. However, it is notable that MSCs also secrete moderate levels of IL-6 in the secretome. Therefore, the high levels of IL-6 observed in the protein expression of IL-6 could be a composite of residual IL-6 from the DPD-MSC secretome and induced IL-6 from PBMCs. Additionally, the DPD-MSC secretome decreased IL-6 expression at the transcriptional level, suggesting pleiotropic regulation of the inflammatory response. Because of its role in immune suppression, we were surprised to observe a significant decline in IL-10 levels. Interestingly, the secretion of IL-10 is pleiotropic, and its levels are elevated in response to the increasing pro-inflammatory environment in the host. AA30 is known to act on macrophages and on B- and T-cell lymphocytes and exhibits antiviral activity.

Since we used human stem cells and PBMCs for our experiments, the results can be extrapolated, with suitable caution, to human cases. Our results provide evidence for the mechanistic action of AA30 by having employed human

---

**Fig. 5** (a) Protein levels of IL-1β, IL-10 and IL-6 in PBMCs treated with DPD-MSC secretome, DPD-MSC-CM + LPS and DPD-MSC secretome-AA30 + LPS analysed by the flow cytometry analyzer. Data shown are protein levels in pg/mL ± SD (n = 3, *p < 0.05, **p < 0.01). (b) mRNA expression of IL-6 and IL-1β in the PBMCs treated with DPD-MSC secretome, DPD-MSC-CM + LPS and DPD-MSC secretome-AA30 + LPS analysed by RT-PCR. The data shown are the mean expression ± SD (n = 3, *p < 0.05, **p < 0.01).
cellular models. The outcome of our studies needs to be validated further in pre-clinical models and in clinical trials, including those of COVID-19, using different potencies of AA.

Conclusion

Our results demonstrate that AA30 possesses immunomodulatory potential, as evidenced by the modulated cytokine and growth factor profile in the DPD-MSC secretome in vitro. Furthermore, the DPD-MSC secretome preconditioned with AA30 augmented angiogenesis in a yolk-sac membrane model and ameliorated the LPS-induced cytokine storm in human PBMCs in vitro. The AA30-preconditioned MSC secretome should be further studied in the context of COVID-19 and other inflammatory disorders.

Highlights

- *Arsenicum Album 30* (AA30) is one of the leading homeopathic medicines that has been advocated against SARS-CoV-2 infection.
- Our study demonstrates that AA30 exhibits immunomodulatory potential in vitro and that the AA30-preconditioned secretome of human dental pulp-derived mesenchymal stem cells (DPD-MSC) can augment angiogenesis.
- Potential therapeutic benefits of AA30 and the DPD-MSC secretome in COVID-19, as well as in other inflammatory disorders, should be explored further in clinical models.

Acknowledgements

The authors would like to thank Dr. D.Y. Patil Homeopathic Medical College & Research Centre, Dr. D. Y. Patil Dental College and Hospital and Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, India, for providing infrastructural support for the smooth conduct of the project. We would like to thank Dr. Vini Mehta for her assistance in manuscript editing.

References

Stem Cell Secretome Modulated by AA30C Ameliorates LPS-Induced Cytokine Storm  
Aphale et al.

20 Sanap A, Kheur S, Kharat A, Bhonde R. Ascorbic acid and IFNy pre-conditioning enhance the potency of human mesenchymal stem cells to ameliorate LPS induced cytokine storm. Int Immunopharmacol 2023;122:110643


23 Mizukami A, Swiech K. Mesenchymal stromal cells: from discovery to manufacturing and commercialization. Stem Cells Int 2018; 2018:4083921


32 Clinical Trial Registry of India. Effectiveness of Arsenicum album 30c in prevention of COVID-19 in individuals residing in hot spots of red zones—a multicentric, randomized, cluster level, controlled trial. CTRI/2020/05/025205; 2020


