Secreted factors of human Wharton's Jelly stem cells inhibit growth and biofilm formation of *Staphylococcus aureus in vitro*

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ABSTRACT

Staphylococcus aureus is causing high rates of morbidity and mortality in human. Stem cells secreted factors are safe and efficient alternative to stem cells in stem cells-based therapies. In this study, this study aimed to evaluate the antibacterial activity of factors secreted by human Wharton's jelly mesenchymal stem cells secretome (hWJ-MSCs) against *Staphylococcus aureus*, hWJ-MSCs were cultured and collected their secreted factors to be used for *In vitro* antibacterial screening against *Staphylococcus aureus*. Antibacterial activity, antibiofilm, and minimum inhibitory concentrations (MIC) of hWJ-MSC-S against *Staphylococcus aureus* were determined. The present data showed that hWJ-MSC secreted factors significantly inhibit the growth of two clinical isolate of *Staphylococcus aureus.* hWJ-MSC-secreted factors reduced the growth of *Staphylococcus aureus* by mor than 87% compared to un-treated control. MIC values were 4.68 µg/ml and 1.17 µg/ml for *Staphylococcus aureus 76, and Staphylococcus aureus* 105, respectively. The finding from this work could be utilized to develop an effective therapeutic approach to treat *Staphylococcus aureus* infection.

Keywords: Stem cells, secreted factors, antibacterial, Staphylococcus aureus

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a multi-diseases causing pathogen that causes various diseases in human ranging from minor skin abscesses to life-threatening infections. (Duerden, 2012; Fowler *et al*., 2005). The rate of community and hospital-acquired *S. aureus* infections is steadily rising and become more common, especially a methicillin-resistant *S. aureus* (MRSA) strains (Schaumburg *et al*., 2012). New treatment strategies, as well as an appropriate animal model for testing these therapeutic approaches are urgently required.

S. aureus pathogenicity and infection are mainly based on its wide range of host-targeting virulence factors, most notably its arsenal of seven different Pore-forming toxins (PFTs) (Alonzo & Torres, 2014; Berube & Wardenburg, 2013). PFTs are virulence factors that found in a variety of human pathogens, including *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Staphylococcus aureus* (Alouf, 2003). To date, the best-studied PFTs are those secreted by *S. aureus* in the context of pneumonia, sepsis, and skin and soft tissue infections(SSTI)

(Rasigade & Vandenesch, 2014; Tong *et al*., 2015).

Antibiotic resistance strains have spread rapidly, limiting the medications available to treat chronic infections in clinical practice. The development of a new antimicrobial agent, particularly one that is effective against multidrug-resistant pathogens and/or bacteria living in biofilms, has become increasingly important (Sung et al., 2016). Among other stem cells based antimicrobial agents have shown promises to treat a variety of bacterial infections. Stem cells have strong antimicrobial effects via direct and indirect mechanisms. Indirect antibacterial effects of stem cells are mediated by the secretion of antimicrobial peptides and proteins (Sung et al., 2016; Sutton et al., 2016). The main goal of the present study was to evaluate the antibacterial activity of human Wharton's jelly mesenchymal stem cells secreted factors (hWJ-MSCs-S) against bacterial growth and biofilm formation of *Staphylococcus aureus*.

2. SUBJECTS & METHODS

2.1. Bacterial strains

Staphylococcus aureus 76 and Staphylococcus aureus 105 clinical isolates were used in this study. Bacterial suspensions from each strain were prepared by growing bacteria in nutrient broth. Bacterial suspensions were vortexed to have uniformly distributed, counted, and used appropriately in each experiment.

2.2. Culturing of hWJ-MSCs and collection of their secreted factors

hWJ-MSCs have been revived and maintain in DMEM/F12 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (AA). Cells were incubated at 37 °C and 5% CO2 (Beeravolu et al., 2017). hWJ-MSCs were grown in a complete growth medium to reach 80 % confluency before replacing the culture medium with serum free medium. 48h later, conditioned medium (CM) was harvested, centrifuged at 1000 xg for 10 min to remove cellular residues. Collected CM was stored at - 80°C until used in subsequent experiments. Prior to the experiment, hWJ-MSCs-CM was thawed on ice and protein concentration was determined using bicinchoninic acid (BCA) method.

2.3. Antibacterial activity of hWJ-MSCs-S

Antibacterial activity of hWJ-MSCs-S was evaluated using a broth microdilution susceptibility test, as previously described with some modifications (Bhattacharya et al., 2012; Chen *et al*., 2008; A. Krasnodembskaya et al., 2010; Murray & Hospenthal, 2004). Briefly, serially diluted hWJ-MSCs-S was transferred into wells of 96-well plate in 90µl volume and 10µl of bacterial suspension (104 CFU/ml) was added to each well. Plate incubated at 37°C for 24 h and the optical density (OD) was read at 620 nm using a microplate reader. Positive control wells (bacterial suspension only) and vehicle controls wells (bacterial suspension and SFM) were run in parallel with each assay.

2.4. Determination of minimum inhibitory concentration (MIC)

MIC values of hWJ-MSCs-S against Staphylococcus aureus assessed via using a broth microdilution susceptibility test, as

previously described (Bhattacharya *et al*., 2012; Chen *et al*., 2008; Krasnodembskaya *et al*., 2010; Murray & Hospenthal, 2004). Briefly, two-fold serially diluted hWJ-MSCs-S was transferred into wells of 96-well plate in 90µl volume. The serially diluted concentrations of hWJ-MSCs-S used to determine MIC were 300, 150, 75, 37.5, 18.75, 9.38, 4.68, 2.34, 1.17, 0.58, and 0.29 µg/ml. Subsequently, 10µl of bacterial suspension (104 CFU/ml) was added to each well and plate incubated at 37°C for 24 h before reading the optical density (OD) at 620 nm using a microplate reader. Positive control wells (bacterial suspension only) and vehicle controls wells (bacterial suspension and SFM) were run in parallel with each assay. MIC value was determined as the minimum concentration of hWJ-MSCs-S that significantly decrease OD and inhibit bacterial growth accordingly.

2.5. Antibiofilm assay

The antibiofilm activity of hWJ-MSCs-S was determined using microtiter plate-crystal violet assay as described previously (Khan, Park, Bamunuarachchi, Oh, & Kim, 2021; Zhu et al., 2002). Briefly, serially diluted hWJ-MSCs-S was transferred into wells of 96-well plate in 90µl volume. Subsequently, 10µl of bacterial suspension (104 CFU/ml) was added into the wells and plate incubated at 37°C for 24 h. Then, wells were rinsed with distilled water and stained with crystal In this study we have demonstrated that hWJ-MSCs-S significantly inhibited the growth and biofilm formation of Staphylococcus aureus. At concentrations of 300, 75, and 18.75 µg/ml, hWJ-MSCs-S significantly inhibited the growth of S. aureus 76 **(Figure1A)**, while S. aureus 105 growth was significantly inhibited at 300, violet working solution (0.1 %) at room temperature for 20 min. Excess dye was removed and wells were washed thrice with distilled water. Next, wells were de-stained with 95% ethanol for 45 min and OD of biofilm associated crystal violet was read at 570 nm using a microplate reader. Positive control wells (bacterial suspension only) and vehicle controls wells (bacterial suspension and SFM) were run in parallel with each assay.

2.6. Statistical Analysis

The growth reduction percentage at each treatment was calculated relative to the growth control. Data were showed as means ± standard deviation (SD) of three independent experiments. Comparisons between various treatments were performed by means of Student's t-test and one-way ANOVA. P values < 0.05 was regarded as statistically significant.

3. RESULTS

The urgent need for new strategies and approaches to combat the infection and emergence of multi-drugs resistant bacteria are highly in demand. Antibacterial agents that extracting from natural origin such as plant extracts and stem cells secreted factors appears to be a promising strategy in the fight against pathogens bacteria avoiding side effects associated with synthetic antibiotics.

75, 18.75, and 4.68 µg/ml of hWJ-MSCs-S (Figure1B). In case of S. aureus 76, growth inhibition was 87.17%, and 67.02% and 55.54 at 300, 75, and 18.75 µg/ml concentrations of hWJ-MSCs-S, respectively **(Figure 2A)**. Growth inhibition for S. aureus 105 was 87.25%, 83.21%, and 77.54%, at concentrations 300,

75, and 18.75 µg/ml, respectively (Figure 2B). It is worth mentioning that at the antibacterial effect of hWJ-MSCs-S on S. aureus was noticed at concentration of 300, 75, 18.75, and 4.68 µg/ml. The effect of hWJ-MSCs-S on S. aureus was significantly decreased at the concentration below 4.68 µg/ml (Yagi et al., 2020). MIC value was 4.68 µg/ml and 1.17 µg/ml for S. aureus 76 and S. aureus 105, respectively, **Table 1 & 2**.

Interestingly, there was a variation in biofilm inhibition by hWJ-MSCs-S between the two *S. aureus* isolates. Biofilm formation by *S. aureus* 76 was significantly inhibited by hWJ-MSCs-S at concentrations of 300, 75, 18.75, 4.68 and 1.17 µg/ml (Figure3A). while hWJ-MSCs-S was significantly inhibited the biofilm formation by *S. aureus* 105 at concentration of 300, and 75 µg/ml (Figure3B).

Table 1. OD values of antibacterial activity of hWJ-MSCs-S against *Staphylococcus aureus*

	Concentration (µg/ml)												
	UT	**SFM**	**300**	**150**	**75**	**37.5**	**18.75**	**9.37**	**4.68**	**2.34**	**1.17**	**0.58**	**0.29**
S. aureus 76	0.579 ± 0.105	0.614 ± 0.08	0.063 ± 0.021	0.141 ± 0.015	0.141 ± 0.015	0.186 ± 0.078	0.207 ± 0.036	0.356 ± 0.020	0.399 ± 0.056	0.489 ± 0.021	0.529 ± 0.057	0.545 ± 0.047	0.574 ± 0.065
S. aureus 105	0.532 ± 0.030	0.586 ± 0.038	0.068 ± 0.016	0.083 ± 012	0.088 ± 0.015	0.107 ± 0.010	0.118 ± 0.019	0.148 ± 0.006	0.376 ± 0.047	0.400 ± 0.021	0.412 ± 0.019	0.448 ± 0.018	0.492 ± 0.007

Table 2. Mean MIC Values of hWJ-MSCs-S against *Staphylococcus aureus*

Bacterial isolates	**MIC**
***S. aureus* 76**	**4.68 µg/ml**
***S. aureus* 105**	**1.17 µg/ml**

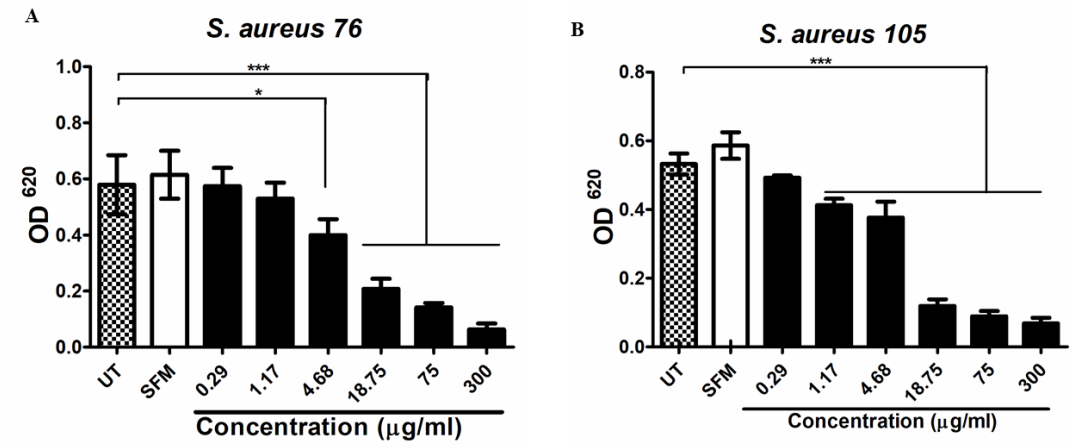

Figure 1: Antibacterial activity of different concentrations of hWJ-MSCs-S against *Staphylococcus aureus.*

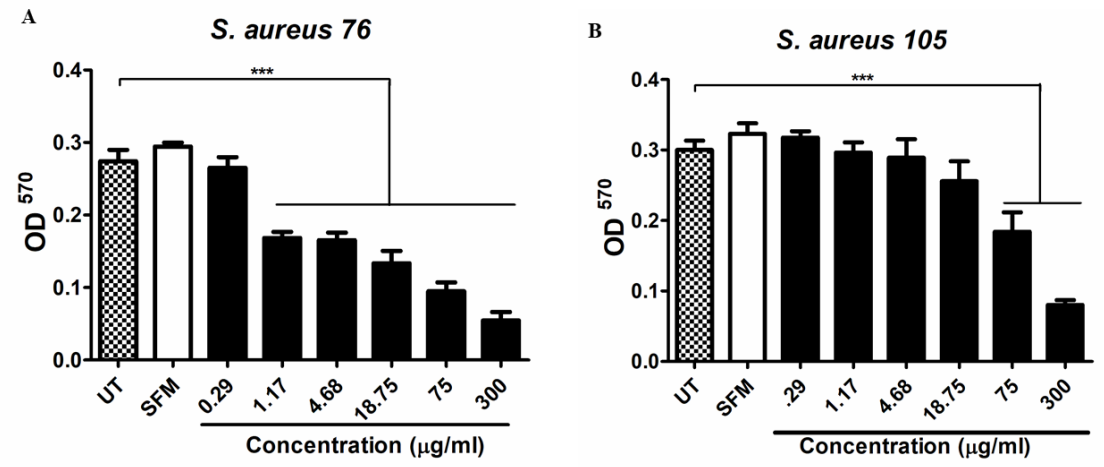

Figure 2: Percentage of growth inhibition by different concentrations of hWJ-MSCs-S against *Staphylococcus aureus*

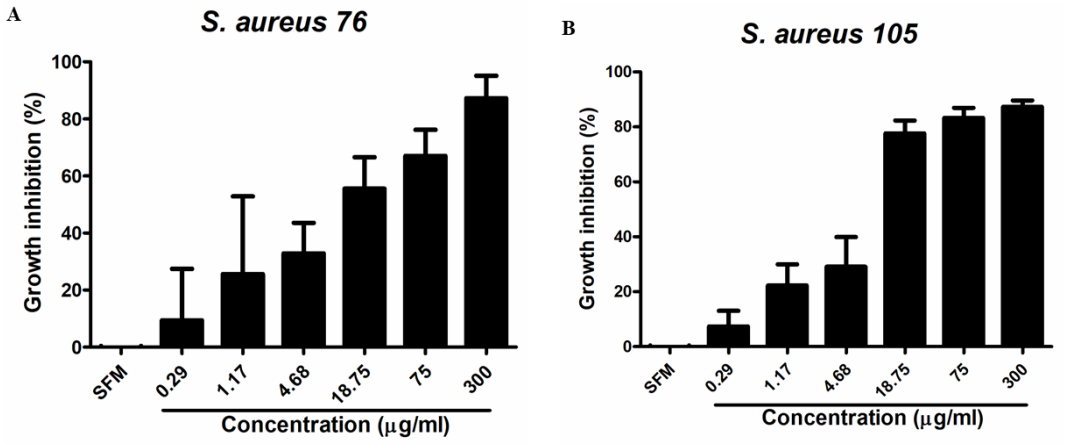

Figure 3: Antibiofilm activity of different concentrations of hWJ-MSCs-S against *Staphylococcus aureus.*

4. Discussion

Results from this work confirm the recent evidence that stem cells have powerful antimicrobial effects through both direct and indirect mechanisms. Secreted factors of stem cells are very rich in antimicrobial peptides and proteins (AMPs). These proteins could inhibit bacterial infection via interfering with inhibiting biofilm formation, increasing phagocyte activity, and enhancing host immune response, particularly in the dynamic coordination of the immune system **(Cortés-Araya *et al.*, 2018; Gupta *et al.*, 2012; Krasnodembskaya *et al.*, 2012; Lee *et al.*, 2013; Raffaghello, Bianchi, Bertolotto, & Montecucco, 2008; Sung *et al.*, 2016; Sutton *et al.*, 2016)**. MSCs have also been discovered to release circular membrane fragments known as microvesicles (MVs), which contain a variety of proteins, mRNAs, microRNAs, and lipids that involve in cell-cell communication and cellular material transfer(Lee et al., 2013). The production of the cationic antimicrobial

peptide LL-37 by MSCs has been reported to be the main mechanism of antimicrobial action **(Anna Krasnodembskaya *et al*., 2010).** LL-37 has antibacterial activity against Gram-negative and Gram-positive bacteria, and it has primarily been studied in vitro using synthetic peptides (**Krasnodembskaya *et al*., 2010; Xhindoli *et al*., 2016) .**

For future research, we can conclude that hWJ-MSCs, or rather their individual antibacterial and antibiofilm components, could be tested in vitro for their biological activity as potent drugs in the eradication of chronic biofilm-associated resistant infections as well as use them in vivo as a new treatment for other invasive infections that are not affected by antibiotics .

All of these findings, combined with their multifunctional properties, open up intriguing perspectives for therapeutic applications of this secretion .

Conclusion

The current study demonstrated the antibacterial effect of hWJ-MSCs-S against S. aureus. hWJ-MSCs-S significantly inhibited the growth and biofilm formation of S. aureus. These data showed that hWJ-MSCs-S inhibits S. aureus growth by more than 87%. Findings from this study could be utilized to develop antibacterial therapeutic against S. aureus using hWJ-MSCs-S-based approaches

5. References

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