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THE NON-PROTEIN FRACTION OF EMBRYONIC STEM CELL SECRETOME EXERTS ANTIBACTERIAL EFFECTS AGAINST ANTIBIOTIC-RESISTANT BACTERIAL STRAINS

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Abstract. In recent years, it has been extremely evident to seek out for new antibacterial agents, because the burgeoning problem of antibiotic resistance and the toxicity of many antimicrobial compounds has forced scientists to turn attention to alternative approaches. Investigating stem cell secretomes, including the non-protein portion, to find new antimicrobials is a promising area in the field. We examined an effect of the non-protein portion within the embryonic stem cell secretome on various bacterial strains, including antibiotic-resistant members. The non-protein fraction of the stem cell secretome was obtained by preparative high-performance liquid chromatography. Bactericidal activity was tested against eight museum bacterial strains and 206 clinical strains by comparing the secretome-related effects on growth of bacterial cultures. The museum strains showed some dose-dependent effects at concentrations of 25–100 μg/ml. Some bactericidal activity was shown at a concentration of 100 μg/ml against the clinical strains of Gram-negative microorganisms of different species, but bacterial sensitivity to the secretome fraction varied, with growth stimulation being detected in some strains. Applying non-protein fraction of the stem cell secretome at higher concentrations of 100–1000 μg/ml showed no dose-dependent effect. The clinical strains of *E. coli* and *P. aeruginosa* were shown to have reduced bactericidal activity after 24-hour incubation. Thus, this study has shown that the non-protein fraction of the embryonic stem cell secretome exerts bactericidal effects against some bacterial strains. However, more detailed studies are needed to identify a mechanism of action and to determine the most effective dose as well as frequency of administration.

Key words: *secretome, embryonic stem cells, antibacterial activity, antimicrobial properties, bactericidal effect.*

НЕБЕЛКОВАЯ ФРАКЦИЯ СЕКРЕТОМА ЭМБРИОНАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК ОБЛАДАЕТ АНТИБАКТЕРИАЛЬНЫМ ЭФФЕКТОМ, В ТОМ ЧИСЛЕ ПРОТИВ АНТИБИОТИКОРЕЗИСТЕНТНЫХ ШТАММОВ БАКТЕРИЙ

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Резюме. В последние годы чрезвычайно актуален поиск новых возможных антибактериальных препаратов, а проблема антибиотикорезистентности и токсичность химиопрепаратов заставляет ученых обращать внимание на альтернативные противомикробные средства. Изучение применения секретома стволовых клеток, в том

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числе его небелковой части, является перспективной областью современной науки. В нашем исследовании было изучено влияние небелковой части секретома эмбриональных стволовых клеток на различные штаммы микроорганизмов, в том числе антибиотикорезистентные. Небелковую часть секретома стволовых клеток получали при помощи препаративной высокоэффективной жидкостной хроматографии. Тестирование бактерицидной активности производилось в отношении 8 музейных штаммов бактерий и 206 клинических штаммов микроорганизмов методом сравнения влияния секретома на рост бактериальных культур. При первичном анализе было выявлено, что на музейных штаммах в концентрации 25–100 мкг/мл исследуемый секретом имеет некоторый дозозависимый эффект. При оценке бактерицидной активности в концентрации 100 мкг/мл в отношении клинических штаммов грамотрицательных микроорганизмов разной видовой принадлежности было показана различная чувствительность штаммов к секретому, причем у некоторых штаммов обнаружена стимуляция роста. Применение более высоких концентраций 100–1000 мкг/мл не выявило дозозависимого эффекта. При этом на клинических штаммах *E. coli* и *P. aeruginosa* было показано снижение бактерицидной активности через сутки инкубации. Таким образом, проведенное исследование показало, что небелковая фракция секретома эмбриональных стволовых клеток также обладает бактерицидным эффектом. Однако требуются более детальные исследования для выявления механизма действия и определения наиболее эффективной дозы и частоты применения.

Ключевые слова: *секретом, эмбриональные стволовые клетки, антибактериальная активность, противомикробные свойства, бактерицидный эффект.*

Introduction

The problem of antibiotic resistance has reached critical proportions [1, 29]. Drugs used to treat diseases caused by antibiotic-resistant strains of microorganisms are often highly toxic, and their effectiveness decreases year after year [20]. Mutations of bacteria under the influence of environmental factors and antibiotics are forcing scientists around the world to search for new drugs that can bypass resistance mechanisms [1, 17].

The use of stem cells in the treatment of a variety of diseases has been widely studied. For example, many studies have confirmed their ability to home in on and integrate into damaged areas of the body, undergo proliferation and repair the tissues [15]. There is evidence that human adipose-derived mesenchymal stem cells (MSCs) when administered systemically can reduce inflammation in colitis and protect against sepsis [9]. In a study by Krasnodembskaya et al. it was shown that human bone marrow-derived MSCs had direct antimicrobial activity, in part due to secretion of the peptide cathelicidin hCAP-18/LL-37 [13]. Several proof-of-concept studies demonstrated that MSCs acted synergistically with current antibiotics to penetrate bacterial biofilms [12], and showed antifungal [2, 39], antiviral [12, 30], and antiparasitic properties [12, 32]. However, the direct application of embryonic stem cells has a number of limitations, due to the risk of teratoma development [33].

The phenomenon of biological regulation through cellular interaction by means of various secreted substances has been actively studied in recent years [40]. It is accepted that the secretome is the sum of molecules and factors secreted by the cell into the external environment [35]. There are obvious advantages to using secreted compounds over stem cells directly because the cells may die or fail to reach the site of action, they cannot be precisely dosed, and there is a risk of tumor formation [19]. The therapeutic potential of the stem cell secretome has been shown for treating a range of diseases, such as myocardial infarction [34], colitis [14], Alzheimer's disease [22] and even alopecia [26]. There is evidence that the secretome has immunomodulatory effects and promotes cell regeneration, migration and proliferation [18]. The protein part of the secretome exhibits anti-inflammatory, antibacterial, antiviral, antifungal, antiparasitic, antioxidant, and angiogenic activities [23, 30]. Studies in recent years have consistently supported the hypothesis that stem cells that have direct antimicrobial activity also produce a secretome capable of inhibiting pathogens. Given the current epidemiological situation with the continuing pandemic, it has been suggested that this secretome may be effective in the treatment of COVID-19-induced pneumonia [19].

However, the compounds responsible for the antibacterial effects of the secretome and the molecular mechanism of their action are still being sought. Some of the effects may be due to the presence of protein factors such as proteases, cytokines, growth factors, etc., but data about the effects of the non-protein secretome fraction is sparse. Thus, the aim of this study was to investigate the antibacterial effect of the non-protein fraction of an embryonic stem cell secretome on various strains of microorganisms, including antibiotic-resistant ones.

Materials and methods

Isolation of cells from embryos and culturing. The source of the cells was an animal embryo before the appearance of histocompatibility antigen (HCA)-containing cells of $CD34^+$ phenotype. Sterile material in 2±0.3 ml of 0.9% sodium chloride was placed in the working chamber of an automatic dense tissue preparation system (Medimachine, Becton Dickinson, USA). Disaggregation was performed

for 30 seconds at 23–25°C. The cell suspension was then removed using a sterile syringe. The working chamber was washed three times with 1 ml of 0.9% NaCl, and the washes were added to the cells. Afterwards, the suspension was filtered through a nylon membrane (Falcon, Becton Dickinson, USA) with a pore diameter of 40 μm. Cells were collected by centrifugation at 400g for 5 min at 25°C and supernatants were discarded.

Cell viability was determined using the vital dye, 7-amino-actinomycin D RUO (7AAD) (Beckman Coulter, USA) on a Cytomics FC500 flow cytometer (Beckman Coulter, USA). Viable cells were scored by registering two parameters, volume/lateral light scattering (SSLin) and fluorescence (7-AAD). Cell phenotype was determined using specific monoclonal antibodies (Beckman Coulter, USA) designed for *in vitro* diagnostics to identify cells expressing CD34 antigen. Only $CD34^+$ cells with a viability of at least 90±5% were selected. Lower viability samples were discarded. Using 0.9% sodium chloride, the concentration of cells in the sample was adjusted to 1.25×10^6 cells per ml. Cells were incubated in culture vials (PP Techno Plastic Products AG, Switzerland) with barriers inside the vials creating a growth surface of 115 cm^2 for 24 hours at 37°C in a 5% CO_2 atmosphere.

Isolation of non-protein stem cell secretome fraction. After one day, the culture supernatants containing secreted products of $CD34^+$cells were collected and lyophilized. The lyophilizates were dissolved in distilled water and the non-protein compounds were separated using an LC-20 Prominence polyblock high performance liquid chromatography system (Shimadzu, Japan) equipped with an LC-AP pump, SIL-10a autodispenser, SPD-20A spectrophotometric detector, TSKgel Amide-80 column (no. 0014460; 10 μm, 21.5 mm ID × 30 cm L) and FRC-10A fraction collector. The sample volume was 5 ml. The mobile phase consisted of distilled water and acetonitrile in a 1:1 volume ratio at a flow rate of 10 ml/min. LabSolution software was used to set up the run parameters to collect the non-protein fractions and reject the protein-containing ones. Subsequent analysis using an Agilent 6850 gas chromatograph equipped with an Agilent 5973N mass-selective detector on a Phenomenex ZB-5MS column (30 m × 250 μm, 0.25 μm) as well as a Milichrom A-02 liquid micro-column chromatograph (Econova, Novosibirsk, Russia) with a Kromasil 100-5-C18 column showed no proteins or peptides in the analyzed fractions. The resulting non-protein fractions secreted from embryonic stem cells were lyophilized, dissolved in distilled water at concentrations of 50, 100, 150, 200, 500, 1000 and 2000 μg/ml and tested for antibacterial activity.

Bacterial strains selected for determination of antibacterial activity of non-protein stem cell secretome products. Bactericidal activity tests were performed against eight museum strains of bacteria and 206 clinical strains of Gram-negative microorganisms highly resistant to antibiotics. The museum strains included both Gram-negative and Gram-positive bacteria: *Escherichia coli* K12 (GISC No. 24036), *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6538P, *S. xylosus* No55/5 (ICVS UrO RAS), *S. epidermidis* No.711 (ICVS UrO RAS), *Micrococcus luteus* var. *lysodeikticus* ATCC 15307. The 120 clinical strains of Gram-negative bacteria highly resistant to antibiotics included 22 strains of *Escherichia coli*, 57 strains of *Acinetobacter baumannii*, 43 strains of *Pseudomonas aeruginosa*, and 84 strains of *Klebsiella pneumoniae*.

Methodology for determining bactericidal activity. We used the method for determining the bactericidal activity of blood serum [15], adapted to test the bactericidal activity of the different concentrations of the fractions. Agar cultures of target bacteria were suspended in isotonic NaCl and counted. Aliquots of 5×10^8 bacteria were introduced into each well of a 96-well plate and 25 μl of secretome fraction at 50, 100, 150 and 200 μg/ml were added to give final concentrations of 25, 50, 75 and 100 μg/ml. Stock solutions of 1000 and 2000 μg/ml were used for the final concentrations of 500 and 1000 μg/ml). For the control, 25 μl of isotonic NaCl alone was added. The cells were incubated for 20 min at 37°C and then 200 μl of meat-peptone broth was added and the plate was incubated for 4 hours at 37°C. The optical density (OD) of the bacterial cultures in the cells was measured using a Multiscan Accent microplate spectrometer (Thermo Electron, Finland) at $\lambda = 492$ nm. Each variant of experiment and control was run with three replicates, and OD readings were averaged.

The bactericidal index (BI, %) was calculated according to the following formula to quantitate the effect of the secretome fraction on the test bacteria:

$$BI = (ODc - ODo)/ODc \times 100\%,$$

where ODc and ODo are the optical densities of control and experimental cultures, respectively. Strains with BI > 10% were considered sensitive. If the OD of the experimental culture exceeded that of the control culture (ODo > ODc), the growth stimulation index (GSI, %) was calculated according to the formula:

$$GSI = ODo/ODc \times 100\%,$$

where ODc and ODo are the optical densities of control and experimental cultures, respectively. Growth stimulation was counted at GSI > 10%. If BI and GSI values were less than 10%, the bacterial strains were classified as indifferent.

The strains of *E. coli* (n = 11) and *P. aeruginosa* (n = 11) that showed relatively high resistance to the bactericidal action of secretome fractions at a concentration of 100 μg/ml, were chosen to evaluate

Table 1. Indices of bactericidal activity of secretome against museum strains of microorganisms (considering a concentration gradient of 25–100 μg/ml) after 4 hours of incubation

Museum bacterial strains studied	BI when using different concentrations of secretome			
	25 μg/ml	50 μg/ml	75 μg/ml	100 μg/ml
***E.coli* K12**	16.5	38.5	50.7	87.9
***E.coli* ATCC 25922**	65.8	75.8	85.0	85.8
***P. aeruginosa* ATCC 27853**	11.1	44.4	77.8	80.0
***S. aureus* ATCC 25923**	79.3	82.6	86.0	86.5
***S. aureus* ATCC 6538P**	54.6	84.5	87.6	88.7
***S. xylosus* No55/5**	61.2	61.4	68.2	68.2
***S. epidermidis* No711**	12.8	20.5	25.6	30.8
***Micrococcus luteus* var. *lysodeikticus* ATCC 15307**	22.6	24.5	30.9	39.2
Median (25–75)	41.90 (16.42–62.48)	52.90 (36.14–84.80)	72.98 (47.66–82.94)	82.92 (82.92–86.77)

the sensitivity to high concentrations of the fraction (100–1000 μg/ml final concentration range) and were incubated for 3, 4, 5 and 24 hours.

Statistical analysis. The analysis and visualization of the data was carried out using the RStudio for MacOS (version 1.3.1056), an open-source software package in the R programming language. Data normality was estimated by the Shapiro–Wilk test. Data were presented as median (25–75) using descriptive statistics (packages "skimr", "psych", "ggpubr", "ggplot2"). To assess the differences between groups, the Kruskal-Wallis test was used, followed by Dunn's post hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

The bactericidal activity of the non-protein part of the stem cell secretome was preliminarily tested on museum strains of microorganisms at concentrations of 25–100 μg/ml. It was found that the secretome fractions exhibited bactericidal activity against all eight museum strains (Table 1). The dose-dependent bactericidal activity was most clearly manifested on *Escherichia coli* K12 and *Pseudomonas aeruginosa* ATSS 27853 than on the other bacterial strains studied, and the highest values of bactericidal activity of the secretome (at 100 μg/ml concentration) were recorded with Gram-negative bacteria *(E. coli, P. aeruginosa)* as well as with strains of *S. aureus*. Coagulase-negative staphylococci *(S. xylosus* and *S. epidermidis)* and micrococcus showed less sensitivity.

Since the highest bactericidal activity was found for a concentration of 100 μg/ml, we additionally evaluated the bactericidal activity at a concentration of 100 μg/ml against clinical strains of Gram-negative bacteria of different species (Table 2). Among the clinical strains studied, it was found that a significant proportion of the isolates exhibited sensitivity to the bactericidal action of secretome fraction. Thus, the range of BI variation was rather wide: from 12.1 to 89.1% for *E. coli*, from 11.0 to 94.7% for *A. baumannii*, from 12.0 to 78.2% for *P. aeruginosa*, and from 10.5 to 100.0% for *K. pneumoniae*. When comparing BI in susceptible strains, no significant differences were found between the clinical strains of *E. coli*, *A. baumannii* and *P. aeruginosa*. However, there was a significant difference in the BI between *P. aeruginosa* and *K. pneumoniae* ($p = 0.0043$). The highest sensitivity was found for *K. pneumoniae* strains, 95.2%. Some strains (from 3.6 to 32.6%) were indifferent to the action of the fractions and some

Table 2. Bactericidal activity (BI) values (concentration — 100 μg/ml) against clinical strains of Gram-negative microorganisms of different species after 4 hours incubation

	Sensitivity parameters			Resilience/Resistance parameters			
Clinical bacterial strains studied	**Proportion of susceptible strains (%)**	**BI range (min-max, %)**	**BI value in susceptible strains (%)**	**Proportion of indifferent strains (%)**	**Proportion of strains with growth stimulation (%)**	**GSI range (min-max, %)**	**GSI value (%)**
***E. coli* (n = 22)**	72.7	12.1–89.1	58.4 (26.5–68.4)	9.1	18.2	16.7–36.7	22.7 (18.1–29.3)
***A. baumannii* (n = 57)**	61.8	11.0–94.7	53.0 (16.4–75.5)	12.3	26.3	10.0–54.3	19.0 (14.3–24.5)
***P. aeruginosa* (n = 43)**	48.8	12.0–78.2	31.7 (18.0–40)	32.6	18.6	10.0–31.6	19.2 (15.7–21.3)
***K. pneumoniae* (n = 84)**	95.2	10.5–100.0	56.4 (31.9–85.6)	3.6	1.2	–	31.8

Note. Data are presented as median (25–75).

actually showed an increase in growth (up to 26.3% in *A. baumannii*).

The bactericidal activity of the non-protein secretome was assessed at higher concentrations (100–1000 μg/ml), and it was found that after 4 hours of incubation the sensitivity was slightly higher for *E. coli* than for *P. aeruginosa* at all concentrations tested (Fig. 1). Significant differences were found between the groups studied at 250 μg/ml ($p = 0.019$) and 500 μg/ml ($p = 0.039$). At the same time, no statistically significant differences were found when using 100 and 1000 μg/ml. It should be noted that in the range of concentrations studied (100–1000 μg/ml), the secretome showed no clear dependence of bactericidal effect on concentration. However, an additional study measuring bactericidal activity indices over a wider incubation time from 3 to 24 hours showed that after 24 hours, all secretome concentrations exhibited very low activity (Fig. 2). A significant difference between 3 and 24 hours of incubation ($p = 0.0034$) was detected in the clinical *E. coli* strains at a concentration of 100 μg/ml. At a concentration of 250 μg/ml a significant difference was found between 3 and 24 hours ($p = 0.0040$), and 4 and 24 hours ($p = 0.0062$). The same differences were shown for 500 μg/ml concentration: 3 and 24 hours ($p = 0.0402$), 4 and 24 hours ($p = 0.0366$); and 1000 μg/ml: 3 and 24 hours ($p = 0.0084$), 4 and 24 hours ($p = 0.0051$). Interestingly, there was no dose-dependent effect: the BI's at different concentrations of secretome at each time interval did not differ significantly from each other, maintaining a tendency for the activity to decrease by 24 hours of incubation at all concentrations (Fig. 2) In a model of clinical strains of *P. aeruginosa* there was also no dose-dependent effect: the bactericidal activity indices studied at different concentrations of secretome at each time interval did not differ significantly from each other at any concentration (Fig. 1). In evaluating the temporal differences at the 100 μg/ml con-

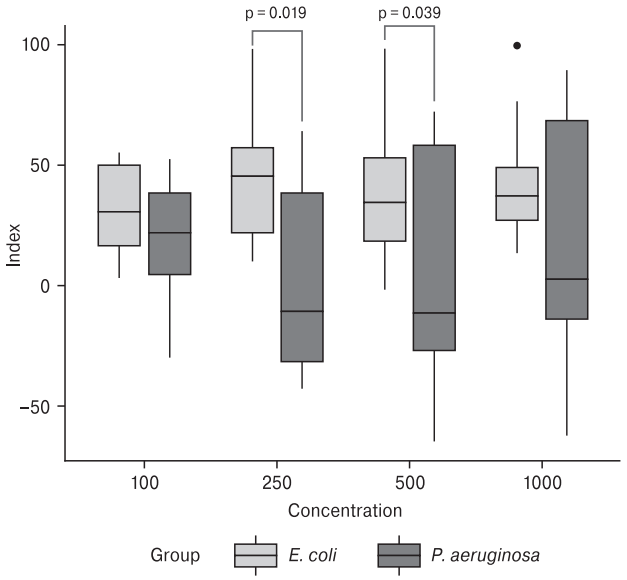

Figure 1. Indices of the bactericidal activity of secretome against *E. coli* and *P. aeruginosa* (considering a concentration gradient of 100–1000 μg/ml) after 4 hours of incubation
Note. An index value below 0 indicates a stimulating effect, index levels of 0–10 indicate an indifferent strain.

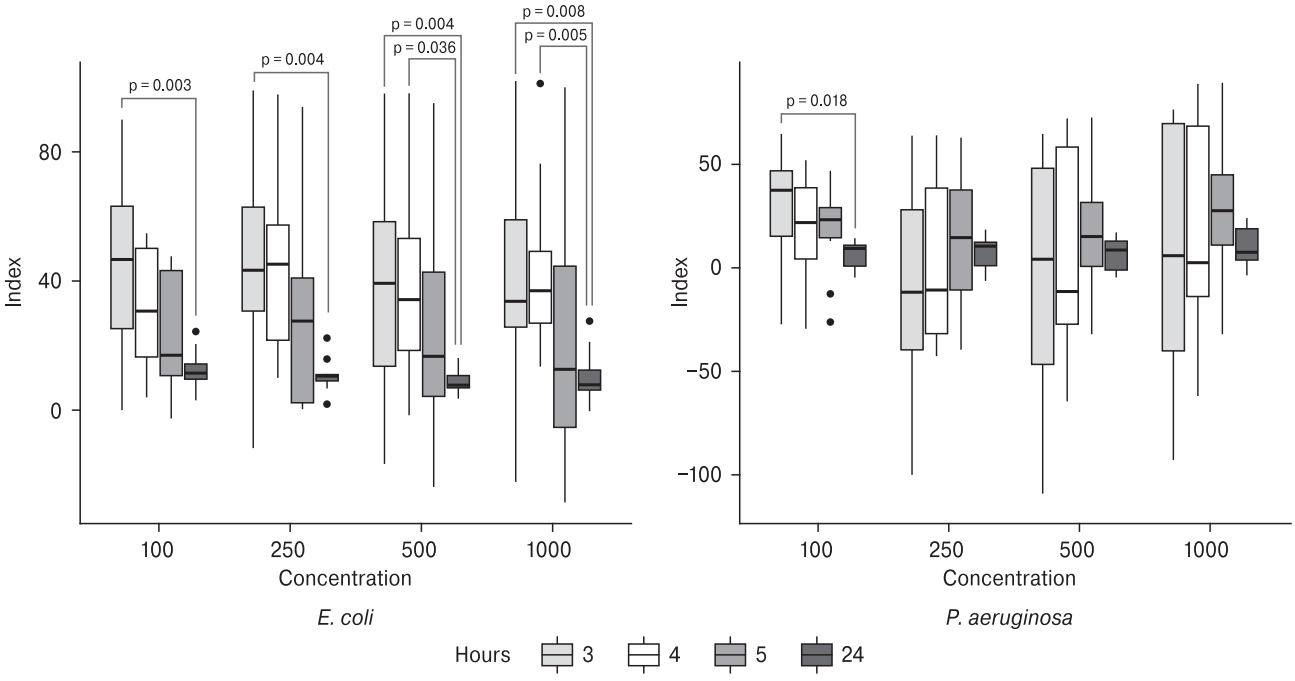

Figure 2. Indices of the bactericidal activity of secretome on clinical strains of *E. coli* (n = 11) and *P. aeruginosa* (n = 11) (considering a concentration gradient of 100–1000 μg/ml)
Note. An index value below 0 indicates a stimulatory effect, index levels of 0–10 indicate an indifferent strain.

centration, a significant difference was observed between 3 and 24 hours of incubation with the fraction ($p = 0.018$).

Discussion

This study showed a bactericidal effect of the non-protein part of the embryonic stem cell secretome on various museum and clinical strains of bacteria. Studies in recent years on the feasibility of using different stem cell secretomes have also shown similar results [3, 31, 37]. Bahroudi et al., showed that the secretome of unstimulated mesenchymal stem cells of bone marrow origin had a significant dose-dependent antibacterial effect against *Vibrio cholerae* [3]. In a study by Saberpour et al., nanocapsules containing MSC supernatant were effective against multidrug-resistant *V. cholerae* [31]. A direct antibacterial effect of MSCs against *Staphylococcus aureus* has been shown by Yagi et al. [37]. One interesting observation in our study was the absence of a dose-dependent bactericidal effect at concentrations above 100 μg/ml, although a significant reduction in action was detected at 24 hours of incubation. Currently, there are no data in the literature on the effect of lyophilized secretome at different concentrations and incubation times on pathogenic microbes, but there is evidence showing the efficacy of lyophilized MSC secretome at a single concentration in a gel with hyaluronic acid and chondroitin sulphate in the treatment of corneal injuries [13]. In a study by Peng et al., MSC supernatant lyophilizate at a concentration of 50 mg/kg was administered endotracheally to mice in an acute lung injury model [12, 27].

Metabolic profiling of the stem cell secretome using MALDI-TOF-MS and other methods resulted in the identification of proteins, lipids and other metabolites [8]. Biologically active substances in cell secretions can include lipids, proteins, nucleic acids, organic acids and cell metabolites, various cytokines, chemokines, growth factors and hormones as well as extracellular vesicles including exosomes (30–150 nm) and microvesicles (100–1000 nm) [10, 16]. However, the literature shows that the secretome's antibacterial effect is mainly attributable to its protein part [16]. A study by Nakashima and colleagues using LC-MS/MS identified about 2400 different proteins in the secretome of human MSCs isolated from umbilical cord matrix, including some associated with immune and viral responses [24]. Marx et al. showed that the MSC secretome inhibited biofilm formation of methicillin-resistant *Staphylococcus aureus* through the action of proteases [21]. Some *in vitro* studies suggested that the antibacterial effect of secretome may be due to the action of chemokines, which, however, are proteins [35, 37, 38].

Some previous studies have explored non-proteomic methodologies to investigate and characterize the non-protein components of the stem cell secretome [26]. One important analysis used nucleotide-based microarrays to identify microRNAs (miRNAs). MiRNAs are an emerging group of non-coding RNAs that play a key role in the regulation of gene expression by interacting with mRNA targets [9]. There is an increasing body of evidence that supports the role of miRNA's in stem cell regeneration [25, 28, 36]. Thus, elucidating the mechanism of the antimicrobial action of the non-protein part of the stem cell secretome identified in this study requires further investigation. This may entail further innovations in bioinformatics and improvements in search algorithms to allow researchers to characterize the non-protein components of the stem cell secretome in greater detail.

As these results have shown, there may be some limitations to the use of secretome, as growth stimulation was detected in some of the strains studied, especially with high concentrations of lyophilizate. Also, some strains were found to be more sensitive (95% of the clinical strains of *Klebsiella*, for example) while in other strains, the percentage of nonresponsive strains or strains with growth stimulation was as high as 50%. This fact requires further investigation using *in vivo* animal models to rule out possible adverse effects.

Conclusion

This study demonstrated the efficacy of the non-protein part of the embryonic stem cell secretome against a wide spectrum of bacterial strains, including antibiotic-resistant ones. The lyophilized secretome showed a different effect depending on the concentration in the range of 25–100 μg/ml, but further increases in concentration showed no dose-dependent effect, and even growth stimulation was found in some strains. Clinical strains of *E. coli* and *P. aeruginosa* showed reduced bactericidal effects after one day of incubation. However, more detailed studies are needed to identify the mechanism of action of the non-protein secretome compounds and to determine the most effective dose and frequency of administration.

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