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Comparative Antimicrobial Effects of Rat Blood-derived Products and Adipose-derived Mesenchymal Stem Cell Conditioned Medium

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ABSTRACT

Background: The antimicrobial characteristics of biological products are fundamental in medicine due to their ability to treat microbial infections. This research aimed to assess and compare the antimicrobial properties of various platelet concentrates, including platelet-rich plasma (PRP), platelet-rich fibrin (PRF), plasma-rich in growth factor (PRGF), and conditioned medium (CM) derived from adipose-derived mesenchymal stem cells (ADSCs) obtained from rats.

Materials and Methods: This experimental study obtained blood-derived products from 50 healthy Wistar rats. After obtaining 5 mL blood sample from each rat via cardiac puncture, it was used to prepare PRGF (group 1), PRP (group 2), and PRF (group 3). For the preparation of PRGF, 0.9% mL of 3.8% sodium citrate was used for every 8.1 mL of the blood. The centrifugal speed was 2500 rpm for 8 min. For the preparation of PRP, the centrifugal speed was 1800 rpm for 10 min and then 3600 rpm for 10 min. PRF was obtained with a centrifugation method of 3000 rpm for 10 min immediately after blood collection, and no anticoagulant was used. Furthermore, 2–4 g abdominal adipose tissue from each rat was used to isolate and culture ADSCs. Then, the CM derived from ADSCs was obtained (group 4). Bacterial strains were grown in blood agar medium and were separately treated with these four groups for 24 h at 37 ˚C.

Results: In this study, PRP stopped the growth of the *Staphylococcus epidermidis* bacterium, with a clear zone of inhibition around the PRP. This blood-derived product also stopped the growth of *Escherichia coli*, while the PRF prevented the growth of three bacteria: *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. PRGF and CM showed no antibacterial activity against these bacterial strains.

Conclusion: PRP and PRF can create antimicrobial conditions in vitro due to their secretion of growth factors like platelet factor 4, fibrinopeptide A, and fibrinopeptide B. They can equally be used as treatment options to ameliorate microbial infections, although preclinical and clinical trials are necessary to understand their characteristics and activities better.

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Introduction

Microorganisms, including bacterial and fungal pathogens, are recognized as a significant cause of ailments in healthcare facilities, particularly in cases such as wounds [1]. Commonly identified bacterial culprits responsible for hospital-acquired infections include *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, as well as the fungal species *Candida albicans* [1, 2]. Although numerous therapeutic methods address these infections, no treatment is one hundred percent effective and without side effects [2]. Thus, it is crucial to develop and employ innovative approaches, such as blood derivatives, stem cells, and their conditioned medium (CM). These products have demonstrated high efficacy in treating these conditions [3, 4]. Today, blood-derived products are an essential treatment source for various diseases, including wounds and burns [5, 6]. These therapeutic products are prepared under various centrifugation conditions. In 1998, Marx et al. introduced the first generation, platelet-rich plasma (PRP), initially used for craniofacial bone grafts [7]. PRP is rich in growth factors derived from whole blood through centrifugation to eliminate erythrocytes [8, 9]. With a growth factor concentration 2-9 times higher than whole blood, PRP is employed to hasten therapeutic responses across various medical fields, such as dentistry, orthopedics, and dermatology [10-14]. As an essential source extracted from blood, PRP comprises numerous growth factors and cytokines that effectively stimulate tissue regeneration in soft tissues and joints [5, 15, 16]. PRP has found significant applications in dermatology for treating androgenic alopecia, promoting wound healing, and facilitating skin rejuvenation [17-24]. Furthermore, numerous studies have demonstrated the antimicrobial properties of human PRP against a variety of pathogens, including bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Streptococcus aureus*, *P. aeruginosa*, and *Streptococcus faecalis*, both in vitro and in clinical settings [25, 26]. In addition, two studies examined its antimicrobial effects on *C. albicans*, *Streptococcus agalactiae*, *E. faecalis*, *Streptococcus oralis*, and other bacteria; PRP inhibited the growth of all three strains, but not *P. aeruginosa* [27, 28].

In 1999, Anitua et al. introduced another blood-derived product known as plasma rich in growth factors (PRGF), which belongs to the first generation of blood derivatives, including PRP [29, 30, 31]. The PRGF stands out as vital for both wound healing and organ regeneration since it contains a variety of growth factors and cytokines, which among them are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and transforming growth factor β (TGF-β) [32, 33]. According to one study, PRGF may have potent antibacterial properties under in vitro conditions [32]. So, the action of PRGF against the four staphylococci strains, which reached its peak within the first few hours after application, served as evidence of its antibacterial activity [32]. Consequently, research indicates that this blood product can effectively inhibit the growth of *S. aureus* and *S. epidermidis* strains [32].

Additionally, platelet-rich fibrin (PRF) represents a more recent blood derivative belonging to the second generation of such products [32, 34]. PRF was first introduced by Choukroun et al. in 2001 and is produced by rapidly centrifuging blood samples collected without anticoagulants [34, 35]. Like PRP, PRF contains many growth factors and bioactive proteins contributing to its therapeutic effects. Furthermore, several studies have indicated that PRF possesses antimicrobial properties, attributed to the presence of growth factors with antimicrobial potential, thus offering promise as a novel therapeutic approach for managing bacterial infections [26, 36-41]. For example, according to findings from a research study conducted by Feng et al., leukocyte- and platelet-rich fibrin (L-PRF) and PRF prepared by horizontal centrifugation (H-PRF) both had notable antimicrobial activity against *S. aureus* and *E. coli*, but H-PRF significantly outperformed L-PRF in terms of its antibacterial activity [40].

Adipose-derived mesenchymal stem cells (ADSCs) have emerged as a promising therapeutic option for treating various ailments, including wounds and burns [42-44]. ADSCs can potentially secrete a range of cytokines and growth factors into the culture medium, collectively termed CM [42, 45]. ADSC-derived CM is rich in numerous growth factors and cytokines, which can expedite tissue regeneration and reconstruction in both in vitro and in vivo environments [42, 45, 46]. Several studies have also confirmed the antibacterial properties of ADSC-derived CM, indicating its potential as a novel therapeutic approach for combating bacterial infections [47-50].

The present experiment aimed to compare the antimicrobial properties of rat PRP, PRF, PRGF products, and CM derived from rat ADSCs on the growth of 7 bacterial strains and 1 fungus strain.

Materials and Methods

Experimental animals

We provided a brief overview of essential animal research ethics principles, guidelines for ethical animal experimentation, institutional education on animal research ethics, and growing alternatives to animal studies. In this experimental study, blood-derived products were obtained from 50 healthy male Wistar rats (*Rattus norvegicus albinus*; mean weight of 265 g; 6 weeks old).

Preparation of blood derivative

After obtaining 5 mL venous blood from each rat, it was used to prepare PRGF (group 1), PRP (group 2), and PRF (group 3). For the preparation of PRGF, 0.9% mL of 3.8% sodium citrate was used for every 8.1 mL of the rat blood sample. The centrifugal speed was 2500 rpm for 8 min. For the preparation of PRP, two centrifuged steps were used, including 1800 rpm for 10 min and then 3600 rpm for 10 min. PRF was obtained with a centrifugation method of 3000 rpm for 10 min immediately after blood collection, and no anticoagulant was used.

Preparation of CM derived from rat ADSCs

Adipose tissue was obtained from 10 rats weighing an average of 250 g. Briefly, adipose tissues were rinsed in a phosphate-buffered saline solution and then digested with collagenase I (Sigma, USA), with an incubation condition of 37 ˚C for 45–60 min. Following incubation, the processed specimens were spun at 1800 rpm for 10 minutes. The collected pellet was then cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA), enriched with 10% fetal bovine serum (Gibco, USA), at a temperature of 37 ˚C, under an atmosphere consisting of 5% CO_2 and 95% humidity. Every 3-4 days, the cultivation medium was refreshed. After reaching 80% confluent (Figure 1), cells were passaged using trypsin enzyme. After three passages, the growth medium was gathered and passed through a 0.2-µm filter for immediate use (group 4).

Measurement of platelet and leukocyte counts

The quantity of platelets and leukocytes was assessed in PRP, whole blood, PRF, and PRGF samples using the Sysmex XS-800i Cell Counter. The platelet count, total white blood cell count, and differential counts were evaluated using a completely automated analyzer (CellDyn™ 4000, Abbott Diagnostics, USA), which employs optical and impedance techniques for analyzing all blood specimens.

Measurement of antimicrobial activity

E. coli (ATCC 25922), *S. aureus* (ATCC 33591), *S. epidermidis* (ATCC 49461), *K. pneumoniae* (ATCC 13883), *E. faecalis* (ATCC 29212), *A. baumannii* (ATCC 17978), *P. aeruginosa* (ATCC 9027), and *C. albicans* (ATCC 10231) were purchased from Iranian Biological Resources Center (IBRC) (Tehran, Iran). The disk diffusion method on Mueller-Hinton agar plates assessed the targeted samples' antimicrobial activity by measuring the inhibition zones. Briefly, 75 µL of rat PRP, PRF, PRGF, or CM from rat ADSCs was added to plates (100 mm, QC Lab) containing 8 mL of Mueller-Hinton agar (Hi-Media, REF M173), which were separately inoculated with *E. coli*, *S. aureus*, *S. epidermidis*, *K. pneumoniae*, *E. faecalis*, *A. baumannii*, *P. aeruginosa*, and *C. albicans*.

E. coli, *S. aureus, S. epidermidis*, *K. pneumoniae*, *E. faecalis*, *A. baumannii*, and *P. aeruginosa* bacteria and *C. albicans* fungus mentioned above were evaluated for susceptibility to cefepime (30 µg), ampicillin (10 µg), ampicillin (10 µg), ceftriaxone (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), and nystatin (100 µg) (Padtan Teb Company, Iran) using the Kirby-Bauer disk diffusion method. Guidelines outlined by the Clinical and Laboratory Standards Institute were strictly followed for this protocol.

Statistical analysis

Data analysis was conducted utilizing SPSS software, version 18 (IBM Corp., Armonk, NY, USA) and Microsoft Excel. Statistical significance was determined at a threshold P.

Results

In this study, PRP, PRF, and PRGF were successfully prepared from rat blood samples, and CM derived from ADSCs (Figure 1) was obtained from rat adipose tissue.

After separating PRP, PRF, and PRGF, the mean platelet counts were 1358±63.85, 1469±68.97, and 1567±78.66, respectively (Table 1). The total mean number of PRP, PRF, and PRGF platelets were 4152±91.87, 4767±106.35, and 5179±103.87, respectively (Table 1). The mean platelet volume (MPV) in the blood sample was 4.83±0.21 mm^3, and after the PRP, PRF, and PRGF isolation, the MPVs were 1.4±0.32, 1.28±0.27, and 1.89±0.31 mm^3, respectively (Table 1). The proportions

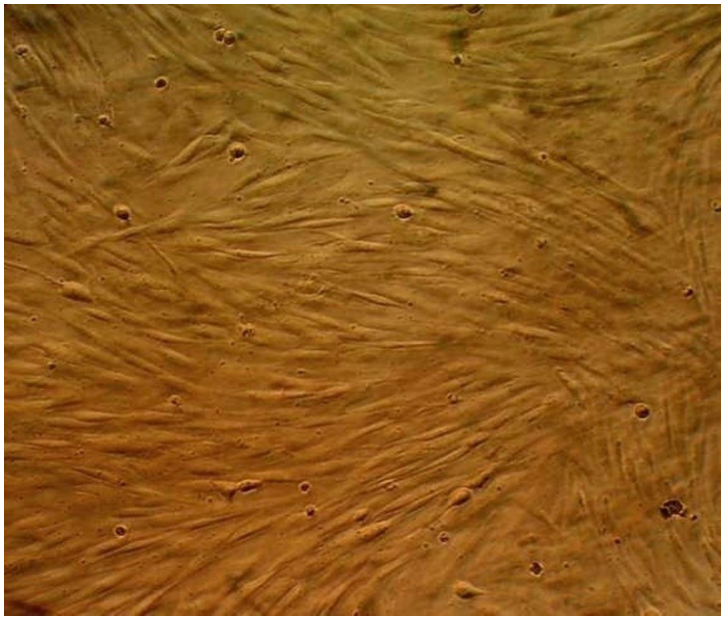

Figure 1. The images of rat ADSCs (captured by an Olympus OLY28 10 CB inverted microscope)

of platelets after processing in PRP, PRF, and PRGF compared to blood samples increased by 2.32±0.16, 2.51±0.29, and 2.681±0.45, respectively (Table 1). The mean leukocyte count in the blood sample was 7.8±0.74×10^3/mm^3, which in PRP, PRF, and PRGF were 0.25±0.43, 0.31±0.57, and 0.52±0.51×10^3/mm^3, respectively (Table 1).

Rat PRP blocked the growth of *E. coli* and *S. epidermidis*, with a clear zone of inhibition around the PRP (Figures 2, 3 and Table 2). At the same time, the rat PRF prevented the growth of three bacteria: *K. pneumonia* (Figures 3 and 4a, and Table 2), *S. epidermidis* (Figures 3, 4b and Table 2), and *P. aeruginosa* (Figures 4c and 3, and Table 2). Rat PRGF and CM derived from rat ADSCs showed no antimicrobial activity against these bacterial strains (Figure 3).

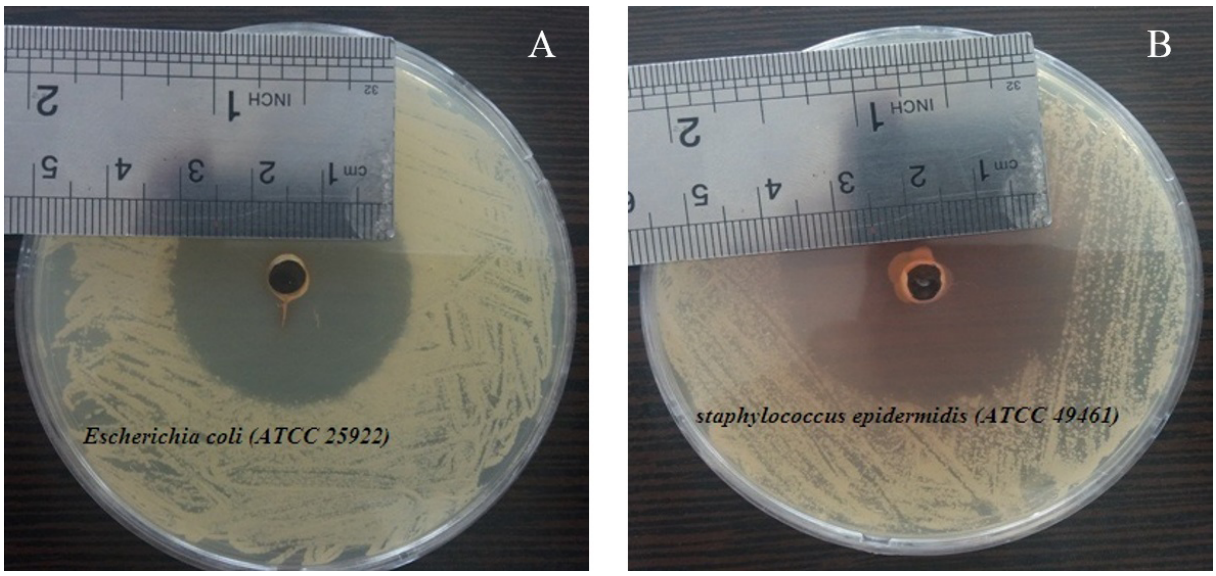

Figure 2. Zone of inhibition seen around PRP in growth medium of *E. coli* (a) and *S. aureus* (b) bacteria

Table 1. Composition and characteristics of the different blood products (PRP, PRF, and PRGF obtained from rat blood)

Group	Mean±SD				
	Platelets (10^3/mm^3)	Total Number of Platelets	Mean Platelet Volume (mm^3)	Platelet Enrichments	Leucocytes (10^3/mm^3)
Whole blood	585±57	-	4.83±0.21	1±0	7.8±0.74
PRP	1358±63.85	4152±91.87	1.4±0.32	2.32±0.16	0.25±0.43
PRF	1469±68.97	4767±106.35	1.28±0.27	2.51±0.29	0.31±0.57
PRGF	1567±78.66	5179±103.87	1.89±0.31	2.681±0.45	0.52±0.51

Abbreviations: PRP: Platelet-rich plasma, PRF: Platelet-rich fibrin, PRGF: Plasma rich in growth factor.

Discussion

Blood derivatives are biological products that are used in medicine today. Several studies have confirmed the antimicrobial properties of blood derivatives [36, 37, 51].

Moreover, in vitro findings suggested that platelets could release proteins reflecting antimicrobial features versus fungi and bacteria [26, 36, 37]. Other mechanisms used in antimicrobial activity by blood derivative products have been reported as reactive oxygen species production and binding and internalizing to microorganisms [27, 52, 53].

Earlier research has shown that certain blood derivatives, such as PRP, PRF, and PRGF, can effectively hinder the growth of various microorganisms, including those responsible for periodontal diseases and wound infections [26, 27, 53-55].

For example, Drago et al. documented that pure-PRP may demonstrate appropriate antibacterial activities against microorganisms isolated from the oral cavity, including *E. faecalis*, *C. albicans*, *S. agalactiae*, and *S. oralis* [27].

Similarly, Badade et al. and Yang et al. verified that PRP has antibacterial effects, suggesting the use of this blood derivative as an inhibitory agent for the growth of two important agents of periodontal diseases, *P. gingivalis* and *A. actinomycetemcomitans* [26, 53].

In addition to blood derivatives, the significant progress in stem cell development illustrates various territories of ADSC therapeutical approaches [50].

Table 2. Zone of inhibition in blood products and CM from rat adipose-derived MSCs

Organisms Name	Inhibition Zone Measurements (mm) Encompass the 80 mm Diameter of the Disk			
	PRP	PRF	PRGF	CM Derived From ADSCs
E. coli (ATCC 25922)	29.45	0	0	0
S. aureus (ATCC 33591)	0	29.56	0	0
S. epidermidis (ATCC 49461)	30.41	0	0	0
K. pneumoniae (ATCC 13883)	0	27.61	0	0
E. faecalis (ATCC 29212)	0	0	0	0
A. baumannii (ATCC 17978)	0	0	0	0
P. aeruginosa (ATCC 9027)	0	26.74	0	0
C. albicans (ATCC 10231)	0	0	0	0

Abbreviations: PRP: Platelet-rich plasma; PRF: Platelet-rich fibrin; PRGF: Plasma rich in growth factor; CM: Conditioned medium.

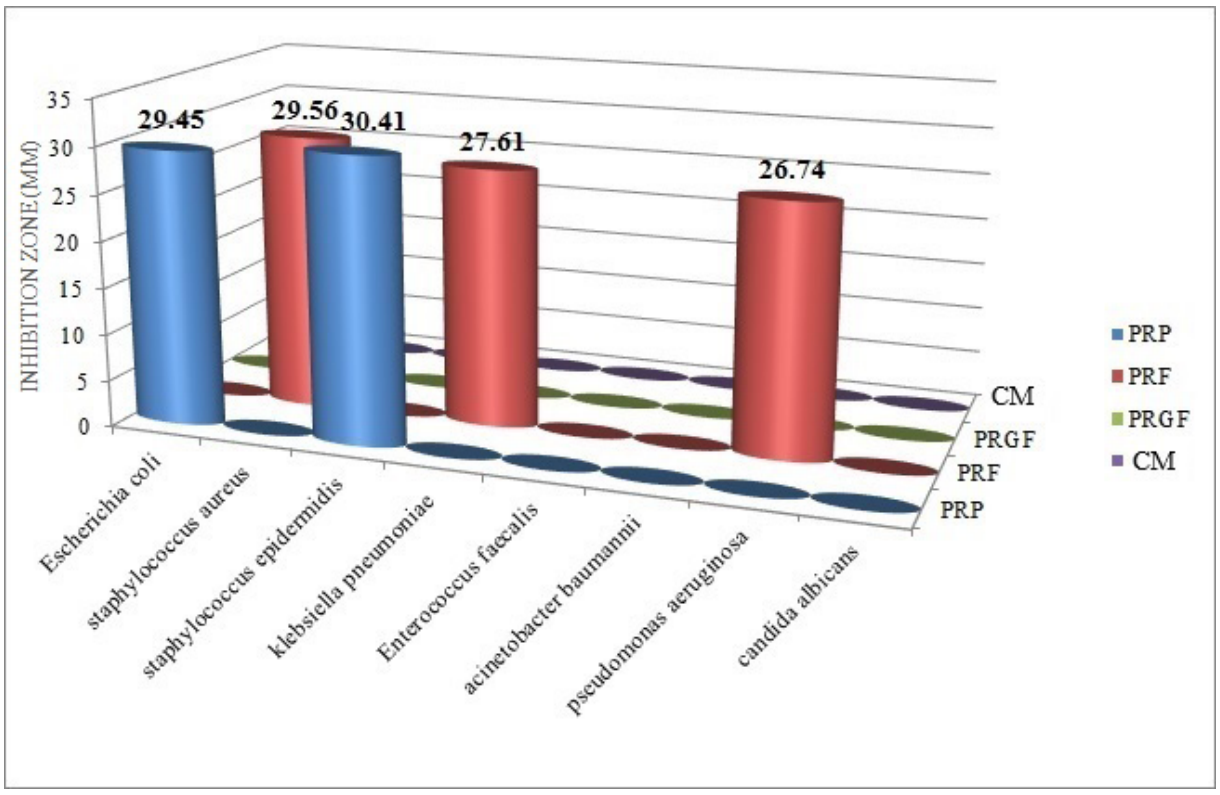

Figure 3. Antibacterial activity of rat PRP, PRF, PRGF, and CM against burn wound pathogens

Abbreviations: CM: Conditioned medium derived from adipose-derived mesenchymal stem cells; PRGF: Plasma rich in growth factors; PRF: Platelet-rich fibrin; PRP: Platelet-rich plasma.

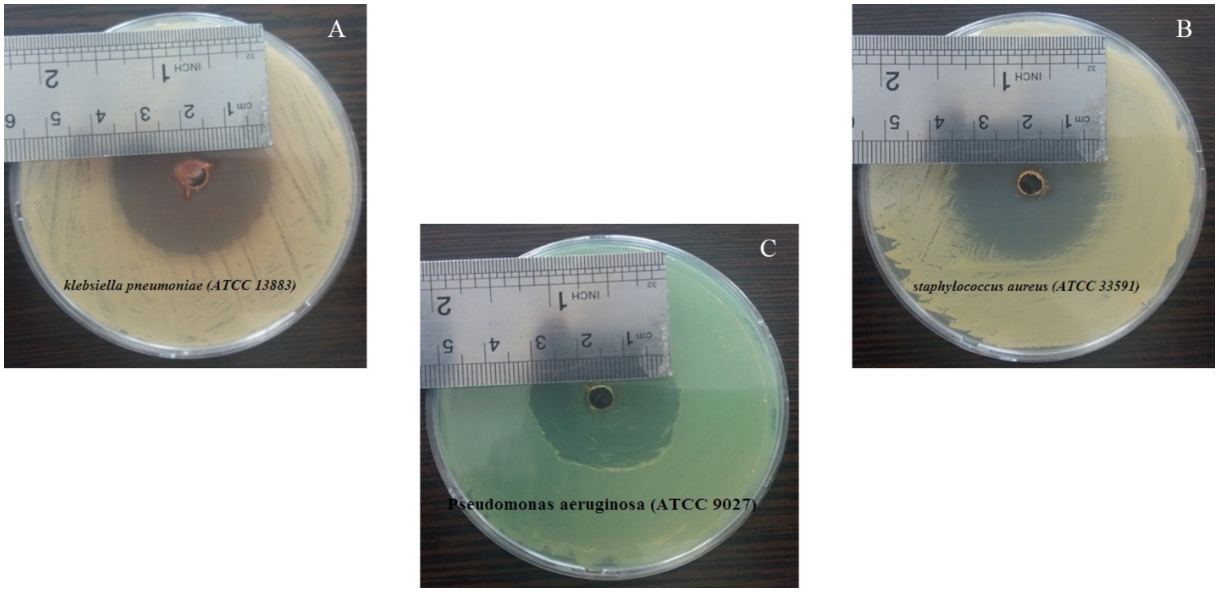

Figure 4. Zone of inhibition around PRF in growth medium of *K. pneumonia (a), S. epidermidis (b) and P. aeruginosa (c)*

Previous studies have strongly suggested the excellent potential of ADSCs and reported the release of growth factors and clinically positive traits [42, 45-50]. Furthermore, ADSC-derived CM encompasses a broad spectrum of growth factors and cytokines that promote tissue regeneration and reconstruction in vitro and in vivo. Moreover, these factors contribute to the observed antimicrobial properties associated with ADSC-derived CM, as demonstrated by multiple studies [42, 45-50].

For example, an experimental study demonstrated that ADSC administration may lead to the repression of *S. aureus* growth in a mice model [50].

Previous studies revealed positive results in the antimicrobial effects of PRP, PRF, and PRGF products and CM derived from ADSCs [26, 50, 53]. This study aims to compare the antimicrobial effects of these products with each other, which is considered a novelty.

We speculate that these blood derivatives may inhibit microbial growth in vitro.

Our rat PRP findings approved some previously published results, as reported by Cieslik-Bielecka that leukocyte and PRP could remarkably inhibit *E. coli* growth [56]. In addition, a clinical study has shown that PRP could help inhibit *S. aureus* and *E. coli* growth, which is consistent with our study [57]. In addition, our rat PRP results agree with some of the findings of Smith et al.. They showed that both activated and inactivated PRP might significantly reduce the development of *S. aureus* and *Staphylococcus epidermis* [58].

Based on our data, the standard strains of *E. coli* and *S. epidermidis* were susceptible to rat PRP, while the remaining strains were resistant to rat PRP. Furthermore, three standard strains of *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* were susceptible to rat PRF, while the remaining strains were resistant to rat PRF. All strains were resistant to rat PRGF and rat ADSC-derived CM.

Furthermore, it seems that the secretion of growth factors with antimicrobial activity by PRP and PRF can inhibit the growth of bacteria by these two blood derivatives.

Although numerous studies demonstrated the antimicrobial activity of human platelet concentration, contradictory findings are obtained in some in vitro studies, whereas the platelet derivative products could have either suppressive or supportive effects on specific bacterial strains. For example, Bielecki et al., similar to our results, demonstrated no human PRP activity against *E. faecalis*. At the same time, four other studies reported a suppressive effect versus the same microorganism [28, 59-61].

Furthermore, our findings are inconsistent with some author reports that introduce human PRP as an effective inhibitor of the growth of *P. aeruginosa* [60, 62-64]. However, some other studies still agree with our findings [29, 32, 65, 66].

Taken together, the present study findings suggest that rat blood derivative products, especially PRF and PRP, could be used as a novel strategy for treating diseases related to pathogens, such as burns and wounds. However, further preclinical and clinical studies are needed to validate disease treatment better.

Conclusion

The current research unequivocally established that both rat-derived PRP and PRF secrete various growth factors involved in antimicrobial activity and tissue healing and are considered a new therapeutic strategy for inhibiting bacterial growth.

Ethical Considerations

Compliance with ethical guidelines

This investigation was approved by the Research Ethics Committees of Islamic Azad University, Qom Branch (Code: IR.IAU.QOM.REC.1402.027).

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Authors contribution's

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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