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# Effectiveness of Wharton's jelly mesenchymal stem cell medium on burn wound healing: Focus on apoptosis, necrosis, and autophagy

## Eficacia del medio de células madre mesenquimales de la gelatina de Wharton en la cicatrización de quemaduras: Enfoque en apoptosis, necrosis y autofagia

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**ABSTRACT**

The aim of this study is to evaluate the treatment efficacy of Platelet–Rich Plasma (PRP), silver sulfadiazine, and Wharton Jelly Mesenchymal Stem Cell–Derived Conditioned Medium (WJ–MSC–CM) on burn wounds using a rat model. The study included four groups, each with 16 rats, and the groups were further divided into two subgroups (n=8) for the 7$^{th}$ and 14$^{th}$ days of the treatment process. Group 1 received no treatment after the burn. Group 2 received PRP (Platelet–Rich Plasma) treatment on the first day after the burn. Group 3 was treated with silver sulfadiazine on the first day after the burn. Group 4 received WJ–MSC–CM on the first day after the burn. In the current study, the expression of Caspase–3, Bcl–2, TNF–α, p21, and Beclin–1 genes among the groups was evaluated by Real–time PCR. The silver sulfadiazine and WJ–MSC–CM treatment groups exhibited lower Bcl–2 expression and higher Caspase–3 and Beclin–1 expression compared to the other groups. TNF–α and p21 expression was high in the burn control group and showed lower expression in the treated groups. The current findings demonstrate that WJ–MSC–CM exhibits healing efficacy on burn wounds comparable to the reference drug (silver sulfadiazine) by inducing apoptosis and autophagy and reducing necroptosis and DNA damage. Additionally, PRP provided some positive benefits compared to the control group but was less effective than the other treatments.

**Key words:** Apoptosis; autophagy; burn wounds; Wharton jelly mesenchymal stem cell–derived conditioned medium

**RESUMEN**

El objetivo de este estudio es evaluar la eficacia del tratamiento con Plasma Rico en Plaquetas (PRP), sulfadiazina de plata y Medio Condicionado Derivado de Células Madre Mesenquimales de Gelatina de Wharton (WJ–MSC–CM) en heridas por quemaduras utilizando un modelo animal. El estudio presentado consistió en 4 grupos, cada uno con 16 ratas, y los grupos se dividieron además en dos subgrupos (n=8) para los días 7 y 14 del proceso de tratamiento. El Grupo 1 no recibió ningún tratamiento después de la quemadura. El Grupo 2 recibió tratamiento con PRP (Plasma Rico en Plaquetas) el primer día después de la quemadura. El Grupo 3 fue tratado con sulfadiazina de plata el primer día después de la quemadura. El Grupo 4 recibió WJ–MSC–CM el primer día después de la quemadura. En el estudio actual, la expresión de los genes Caspasa–3, Bcl–2, TNF–α, p21 y Beclin–1entre los grupos se evaluó mediante PCR en tiempo real. Los grupos de tratamiento con sulfadiazina de plata y WJ–MSC–CM mostraron una menor expresión de Bcl–2 y una mayor expresión de Caspasa-3 y Beclin–1en comparación con los otros grupos. La expresión de TNF–α y p21 fue alta en el grupo de control de quemaduras y mostró una menor expresión en los grupos tratados. Los hallazgos actuales demuestran que WJ–MSC–CM presenta una eficacia de curación en las heridas por quemaduras comparable al medicamento de referencia (sulfadiazina de plata) al inducir apoptosis y autofagia y reducir la necroptosis y el daño del ADN. Además, el PRP proporcionó algunos beneficios positivos en comparación con el grupo de control, pero fue menos eficaz que los otros tratamientos.

**Palabras clave:** Apoptosis; autofagia; heridas por quemaduras; medio condicionado derivado de células madre mesenquimales de la gelatina de Wharton

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## INTRODUCTION

Thermal burn injuries are wounds caused by heat that occur in the skin. Burn injuries can significantly affect the skin and lead to severe complications that may result in death [1]. Burns are associated with approximately 15% of global human deaths annually [2]. In animals, the incidence of burn injuries has increased in recent years due to factors such as forest fires and farm fires [3, 4, 5]. Burns can lead to severe complications such as shock, infections, electrolyte imbalances, and respiratory failure [6].

Burn treatment can be approached through various methods. One of the most critical components of topical wound treatment is the use of silver–containing applications. Silver–containing topical treatments significantly reduce the risk of sepsis and death. Silver ions contribute significantly to bactericidal activity by binding to bacterial DNA, while the sulfadiazine component affects bacterial metabolic processes. Consequently, such treatments are considered the gold standard in burn management [7, 8]. Another treatment option is Platelet–Rich Plasma (PRP), which accelerates wound healing, reduces infection rates, and is easily accessible [9]. The effects of PRP on burn healing have been demonstrated in numerous studies [10, 11, 12].

Apoptosis, or programmed cell death, is the process by which cells systematically disassemble into component parts that will later be ingested and removed [13]. Caspase–3 is known as a major effector caspase and is described as the primary mediator of apoptosis. Caspase–3 can be activated by both extrinsic (death ligands) and intrinsic (mitochondrial) signaling pathways. The anti–apoptotic protein Bcl–2 is located on the outer mitochondrial membrane and prevents the release of apoptogenic factors from the mitochondria into the cytosol [14, 15, 16, 17].

Autophagy involves the sequestration of intracellular macromolecules and organelles within a membrane, which then fuses with lysosomal enzymes for degradation. The most distinct morphological feature of this type of cell death is the presence of vesicles containing cytoplasmic fragments or intracellular organelles such as mitochondria and endoplasmic reticulum (ER), surrounded by a double–membrane structure. Beclin–1, located in the endoplasmic reticulum, mitochondria, and perinuclear space, plays a role in the formation of autophagosomes essential for autophagy [13].

Necroptosis is a form of programmed cell death that mechanistically resembles apoptosis but differs from necrosis and apoptosis in terms of its mechanistic and biochemical pathways [18, 19]. Necroptosis can be induced by receptors that trigger apoptosis, such as the TNF receptor superfamily (FasL, TNF$\alpha$, TRAIL, etc.), TLRs, and interferon receptors [20, 21]. Singer and McClain [22] reported an increase in necrosis, particularly at the 24$^{th}$ and 48$^{th}$ hours following thermal burns in rats (*Rattus norvegicus)*, and suggested that necrosis plays a more significant role than apoptosis in the progression of thermal burn wounds.

P21, also known as Cyclin–Dependent Kinase Inhibitor 1, is a protein that halts the cell cycle by inhibiting cyclin–dependent kinases [23]. Initially discovered as a tumor suppressor and cyclin–dependent kinase (CDK) inhibitor, p21 plays a crucial role in halting cell division in response to cell–toxic agents like DOX that cause DNA damage [24, 25]. Excessive expression of p21 has been shown to result in the arrest of the cell cycle at the G1, G2, or S phases [26, 27, 28, 29]. Conversely, cells lacking p21 do not pause the cell cycle despite DNA damage [29]. p21 can induce extrinsic apoptosis through activation of TNF death receptor pathways or intrinsic apoptosis via upregulation of the pro–apoptotic gene BAX [30].

In the past 20 years, stem cells have been utilized in the treatment of various diseases, including burns [31]. Mesenchymal stem cells, which can be obtained from various tissues and possess self–renewal and multipotent differentiation capacities, are a significant resource for tissue repair. Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various mesodermal cells, including bone, cartilage, and adipose tissue. One source of these cells is the Wharton's Jelly of the umbilical cord. Wharton's Jelly–derived mesenchymal stem cells from humans are often preferred due to their high proliferation capacity and ease of availability [32]. The presented study evaluates apoptosis, necrosis/necroptosis, autophagy, and DNA damage in a rat model of experimental thermal burns following treatment with PRP, silver sulfadiazine, and Wharton's Jelly mesenchymal stem cell–derived conditioned medium.

## MATERIAL AND METHODS

The tissues used in this study were obtained from the project titled "Investigation of the Effectiveness of Wharton's Jelly Mesenchymal Stem Cell–Derived Conditioning Medium on Wound Healing in a Burn Model," which was approved by the Selçuk University Experimental Medicine Application and Research Center Ethics Committee under decision number 2022-24 dated June 24, 2022. The study, identified by project number 22122017, was conducted at the Selçuk University Experimental Medicine Research and Application Center with the ethics approval dated June 24, 2022.

### Animal material

A total of 64 Wistar Albino rats (*Rattus norvegicus*), comprising 32 females and 32 males, each weighing between 300–350 g (Mettler Toledo, Balance XPR204S, Switzerland) were used in the experiment. All procedures were performed under general anesthesia. The rats were acclimatized to the environment for one week under standard laboratory conditions: standard diet, a 12–hour light–dark cycle, and appropriate temperature and humidity levels were maintained.

### Determination of experimental groups and burn application

After shaving the dorsal areas of the rats under general anesthesia, a second–degree deep burn model was applied [33]. The homogeneity of the burn wound was ensured using a device that measured and maintained a constant temperature. This allowed burns to be induced in each rat by applying heat at the same temperature. No pressure was applied during the heat application to create the burn. Each rat was placed in a separate cage. For pain management, 2 mg·ml$^{-1}$ acetaminophen was administered via drinking water. The rats were monitored daily post–surgery for overall condition, body weight, signs of malnutrition, and infection. A total of 64 rats were randomly divided into 4 groups: Group 1 (n=16), Group 2 (n=16), Group 3 (n=16), and Group 4 (n=16). Each group consisted of 8 females and 8

males. Each group was further divided into two subgroups: Group 1a (n=8), Group 1b (n=8), Group 2a (n=8), Group 2b (n=8), Group 3a (n=8), Group 3b (n=8), Group 4a (n=8), and Group 4b (n=8). Each subgroup contained 4 males and 4 females. Group 1; received no treatment post–burn. Group 2; received PRP (Platelet–Rich Plasma) treatment on the first day (d) post–burn. Group 3; was treated with Silver Sulfadiazine (1%) on the first d post–burn. Group 4; received Wharton's Jelly Mesenchymal Stem Cell–Derived Conditioned Medium on the first d post–burn. Samples were collected from rats in Groups 1a, 2a, 3a, and 4a seven d after treatment, and from rats in Groups 1b, 2b, 3b, and 4b fourteen d after treatment. Euthanasia was performed via cervical dislocation under general anesthesia on the 7$^{th}$ d for 32 rats and on the 14$^{th}$ d for the remaining 32 rats. The tissues collected from the lesioned areas were preserved at -80°C (ZK Meiling, DW–HL–398S, China) until molecular analysis was conducted.

### Real–time PCR analysis

RNA isolation was performed from the samples collected during necropsy and stored at -80°C using the SanPrep Column microRNA Miniprep Kit (BIO BASIC, USA, Catalog No.: SK881), following the manufacturer's recommendations. cDNA was synthesized from the isolated RNA using the OneScript Plus Synthesis Kit (ABM, Canada, G236, Catalog No.: G236), according to the manufacturer's instructions. Real–time PCR was conducted using the Roche LightCycler 96 (Roche, Switzerland) instrument. For this purpose, ABM BlasTaq™ 2× PCR MasterMix (ABM, Canada, Catalog No: G891) was used. The primer sequences utilized in the study are provided in TABLE I. Separate reaction mixtures were prepared for each gene of interest. For each sample, 15 µl of reaction mixture and 5 µl of cDNA sample were added to the capillaries. The real–time PCR conditions included: 180 s of pre–incubation at 95°C, 15 s of denaturation at 95°C, 60 s of annealing at 60°C, 3 s of extension at 72°C, and 30 s of cooling at 40°C. The expression levels of the genes investigated were calculated using the $2\Delta\Delta^{Ct}$ (Delta Delta Ct) method [34, 35].

### Statistical analysis

Parametric data obtained from molecular analyses were evaluated using the SPSS 22.0 (IBM SPSS Statistics 22, USA) statistical program with One–Way ANOVA and post hoc Duncan test. The data were presented as mean ± standard error (mean±SE). A *P*–value of less than 0.05 was considered the threshold for statistical significance.

This study, by demonstrating the effectiveness of both current methods used in burn treatment and innovative biological treatment options, may contribute to the development of more effective treatment strategies in clinical applications.

***TABLE I***
**Sequences of primers used**

| Primer | Primer Sequence 5′-3′ |
|---|---|
| Bcl–2 | F: 5′–GCAGCTTCTTTCCCCGGAAGGA–3′<br>R: 5′–AGGTGCAGCTGACTGGACATCT–3′ |
| Caspase–3 | F: 5′–GGTATTGAGACAGACAGTGG–3′<br>R: 5′–CATGGGATCTGTTTCTTTGC–3′ |
| TNF–α | F: 5′–GTCGTAGCAAACCACCAAGC–3′<br>R: 5′–TGTGGGTGAGGAGCACATAG–3′ |
| p21 | F: 5′–TTGCACTCT GGTGTCTGAGC–3′<br>R: 5′–AATCTGTCAGGCTGGTCTGC–3′ |
| Beclin–1 | F: 5′–AGCACGCCATGTATAGCAAAGA–3′<br>R: 5′–GGAAGAGGGAAAGGACAGCAT–3′ |
| β–actin | F: 5′–GGCCACAATGGCTGACCATTC–3′<br>R: 5′–AAGGTGACAGCATTGCTTC–3′ |

## RESULTS AND DISCUSSION

In the statistical analysis of Bcl–2 levels, no significant differences were observed between the burn control group at the 1$^{st}$ week PRP and the 2$^{nd}$ week PRP. However, significant differences were found between these groups and the other groups. The lowest Bcl–2 expression levels were observed in the group treated with silver sulfadiazine at the 1$^{st}$ week. The results are presented in FIG. 1.

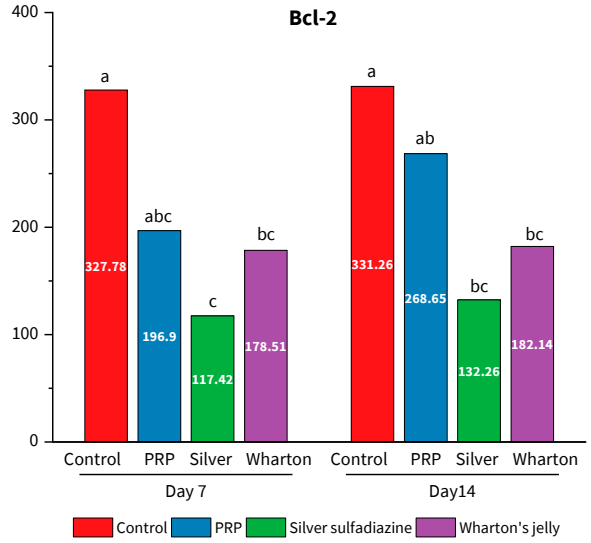


**FIGURE 1. Distribution of Bcl–2 among groups according to Real Time PCR results. [a–c] Different superscripts indicate statistical significance (*P*<0.05)**

This results suggests that silver sulfadiazine and Wharton jelly may induce apoptosis, contributing to the reduction of burn–related tissue damage. Similarly, the lowest levels of Caspase–3 expression were found in the burn control group, while the highest levels were in the silver sulfadiazine and Wharton jelly–treated groups, indicating that these treatments utilize apoptosis to address burn–induced tissue damage.

In the statistical evaluation of Caspase–3, differences were observed between the burn control group at the 1$^{st}$ week and the groups treated with silver sulfadiazine, as well as between the groups treated with Wharton jelly. However, no significant differences were found between the burn control group and the other groups at the 2$^{nd}$ week. The results are presented in FIG. 2.

Although the Caspase–3 expression levels in the burn control group at the 2$^{nd}$ week were about half those in the Wharton jelly–treated 2$^{nd}$ week group, no significant statistical difference

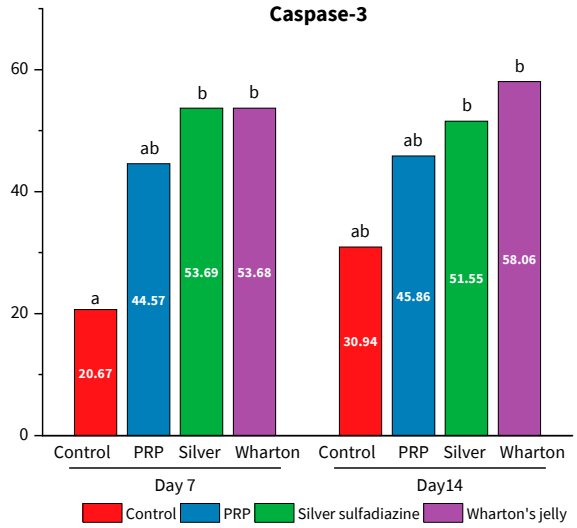


**FIGURE 2. Distribution of Caspase–3 among groups according to Real Time PCR results. [a–b] Different superscripts indicate statistical significance (*P*<0.05)**

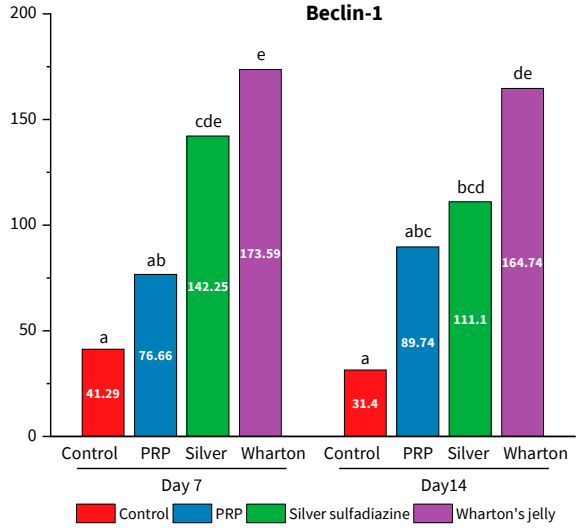


**FIGURE 3. Distribution of Beclin–1among groups according to Real Time PCR results. [a–d] Different superscripts indicate statistical significance (*P*<0.05)**

was found, suggesting that apoptosis begins to increase after the second week post–burn. Tan and Zhang [36] reported that apoptosis increased after the first 6 h in a thermal burn model in rats, while Xiao and Li [33] noted an increase in apoptosis within the first 48 h, followed by a decrease. The present study's findings are consistent with these studies. Additionally, unlike previous studies, this study assessed apoptosis rates on the 7th and 14th days post–burn and found that apoptosis did not decrease after 48 h but rather increased. Gravante and Palmieri [37] noted that the presence and increase of apoptosis could lead to growth in the burn wound. However, in this study, increased apoptosis rates in the burn wounds, following the application of silver sulfadiazine, PRP, and Wharton jelly, suggest that apoptosis plays a crucial role in tissue repair rather than deepening the burn wound. Furthermore, the greater Caspase–3 expression observed in the burn control group at the 2nd week compared to the 1st week suggests that apoptosis is involved in wound healing even in the absence of treatment agents.

In the analysis of Beclin–1, no significant differences were found between the burn control group and the PRP–treated groups. However, differences were observed between the groups treated with silver sulfadiazine and Wharton jelly. The highest Beclin–1levels were observed in the Wharton jelly–treated groups, particularly in the 1st week group, while the lowest Beclin–1levels were found in the burn control group. The results are presented in FIG. 3.

Based on these results, it can be concluded that silver sulfadiazine and Wharton jelly applications might enhance autophagy to address tissue damage and promote regeneration. Autophagy can have dual effects, either reducing or increasing tissue damage. However, in light of other parameters, the higher Beclin–1 levels in the silver sulfadiazine and Wharton jelly–treated groups and the lower levels in the burn control group suggest that autophagy favors cell regeneration and tissue repair. Tan and Zhang [36] reported that autophagy peaked 12 h after a thermal burn in a rat model, while Xiao and Li [33] noted it peaked at 72 h. Similar results were obtained in this study. Additionally, while Bcl–2 expression levels were higher in the 1st week groups compared to the 2nd week groups, this decrease in Bcl–2 levels over time was interpreted as a result of apoptosis.

In the molecular and statistical analyses of TNF–α, it was observed that both the 1st week burn group and the 2nd week burn group were different from all other groups, with the highest TNF–α levels found in the burn control group. The lowest TNF–α levels were observed in the 2nd week group treated with silver sulfadiazine and the 1st week group treated with Wharton jelly. The results are presented in FIG. 4.

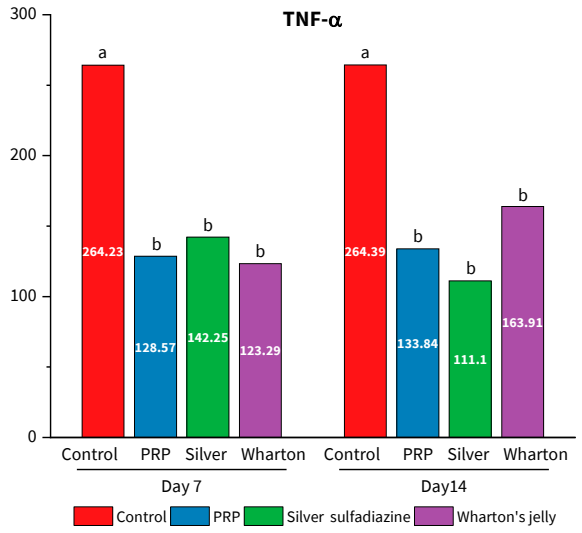


**FIGURE 4. Distribution of TNF–α among groups according to Real Time PCR results. [a–b] Different superscripts indicate statistical significance (*P*<0.05)**

In the investigation of necrosis/necroptosis, Real–Time PCR analysis revealed that TNF–α expression levels were significantly higher in the burn control group compared to other groups, with statistical analysis confirming differences between burn control group and all other groups. Considering the destruction and repair reactions in the burn control, silver sulfadiazine–treated, and Wharton jelly–treated groups, these results are associated with the role of TNF–α in inflammation.

The analyses revealed that the highest p21 expression levels were observed in the burn control group. Differences were noted between the burn control group and the 1st week PRP–treated group, as well as between the burn control group and the silver sulfadiazine–treated groups. The lowest p21 expression levels were detected in the groups treated with silver sulfadiazine. The results are presented in FIG. 5.

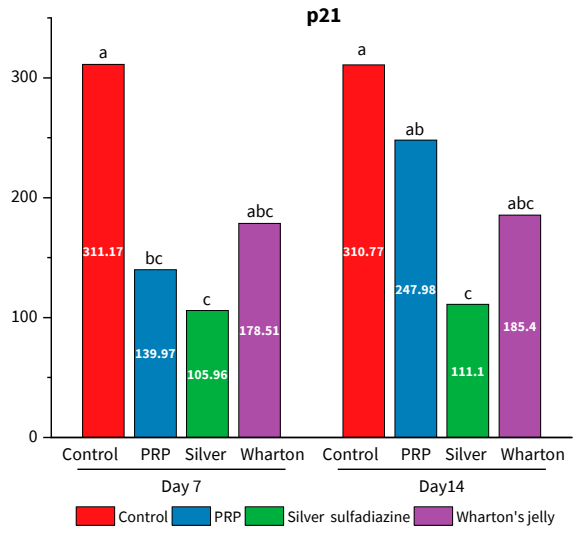


**FIGURE 5. Distribution of p21 among groups according to Real Time PCR results. [a–c] Different superscripts indicate statistical significance (*P*<0.05)**

These results indicate that, as expected, DNA damage was higher in the burn control group, while it was significantly reduced in the silver sulfadiazine groups. Although p21 expression was lower in the Wharton jelly–treated groups, the lack of a statistical difference compared to the control group suggests that Wharton jelly may be less effective or slower in repairing DNA damage compared to silver sulfadiazine.

## CONCLUSION

According to the results of the study, groups treated with silver sulfadiazine and Wharton jelly exhibited higher levels of apoptosis and autophagy, while showing less necroptosis and DNA damage. Although the PRP–treated groups yielded better results compared to the burn control group, no statistically significant differences were observed in apoptosis and autophagy. Although Wharton jelly demonstrated treatment efficacy comparable to that of silver sulfadiazine, its limited practical use and slower treatment response make silver sulfadiazine a more successful option for thermal burn injuries.

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### Conflict of Interests

The authors declare that there is no conflict of interest.

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