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Apoptotic and Cytotoxic Effect of Two Chemical Hemostatic Agents on Human Gingival Fibroblasts (A Comparative *in vitro* Study)

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Abstract

Maintaining moisture control poses a significant challenge in clinical dentistry. While various hemostatic agents have been developed to manage bleeding during dental treatments, limited research has investigated their biological effects on surrounding oral tissues. To evaluate and compare the biological effects of ferric sulfate and aluminum chloride solutions on the Human Gingival Fibroblast cell line (HGF-1). Human Gingival Fibroblasts (HGFs) were allocated into three groups. The control group included the untreated HGFs. The ViscoStat group included HGFs treated with 20% ferric sulfate. The HemoStop group included the HGFs treated with 25% aluminum chloride. Cell viability was assessed after 24 hours of exposure. In addition, apoptosis was evaluated by analyzing *Bax* and *BCL-2* gene expression after 24 hours of exposure utilizing RT-qPCR. Both hemostatic agents depicted decreased cell viability compared to the untreated control. However, HemoStop exhibited higher cell viability compared to ViscoStat. ViscoStat significantly decreased the *mRNA* of *B-cell lymphoma-2* (*Bcl2)* and upregulated *BCL-2-associated X* (*Bax)* expression in HGFs compared to HemoStop. Our data suggest better biocompatibility with HemoStop compared to ViscoStat. In addition, HemoStop depicted significantly decreased genotoxic effect on HGFs compared to ViscoStat.

Keywords: Aluminum Chloride, Ferric Sulfate, cytotoxicity, Human gingival fibroblasts, apoptosis

1. Introduction

In routine clinical dental practice, achieving adequate moisture management is a major difficulty. Positioning restorations at or near the cervical third of the teeth in operative dentistry, or even trauma during cavity preparation, may induce gingival bleeding. Therefore, optimal moisture control is essential to avoid any post-operative complications and treatment failures (Kim et al., 2023).

To manage hemorrhage during dental procedures, a wide array of techniques and hemostatic materials have been developed (Minervini et al., 2024). These approaches aim to control bleeding and facilitate gingival tissue retraction. Common modalities include mechanical methods, chemo-mechanical agents, rotational gingival curettage, and electrosurgery (Lathwal et al., 2022).

Among these, chemo-mechanical approaches are most widely employed, involving the placement of a cord-like material impregnated with chemical agents into the gingival sulcus. These agents typically include chemical compounds that promote tissue shrinkage and gingival retraction (Tarighi et al., 2014). These compounds are indispensable for soft tissue management, particularly in moisture control and gingival retraction during dental procedures. Ideally, such agents should elicit a controlled biological response without causing adverse local or systemic effects (Tabassum et al., 2017).

Chemical retraction agents are classified into two main categories: Class I (vasoconstrictors, such as adrenergic agents) and Class II (astringents or hemostatic agents) (Ayeen et al., 2022). Astringents or hemostatic agents, such as aluminum potassium sulfate, aluminum chloride, ferric sulfate, and zinc chloride, function by inducing protein deposition on mucosal surfaces, thereby enhancing tissue mechanical strength (Chawla et al., 2022). They are particularly effective in controlling capillary and arteriole bleeding. Aluminum chloride and ferric sulfate are favored for their efficacy, ease of application, and minimal tissue trauma (Scarano et al., 2023).

Aluminum chloride is an acidic astringent that achieves its hemostatic effect by contracting tissues in addition to blood proteins precipitation. It has been widely adopted in dental practice due to its efficacy and relatively low cost. However, its low pH raises concerns regarding cytotoxicity and irritation to surrounding tissues (Alzain et al., 2024). It is preferred due to its minimal irritation and reduced systemic side effects. However, it may interfere with the setting of polyvinyl siloxane impression materials. This drawback can be mitigated by rinsing with water (Gupta et al., 2012).

Ferric sulfate is used as a coagulant and hemostatic agent, and it achieves hemostasis by forming a complex of ferric ion-protein that obliterates the capillary openings (Manzoor et al., 2021). It is associated with minimal tissue injury and relatively fast healing. It is most effective when applied directly to bleeding tissues, where it promotes rapid hemostasis through blood coagulation (Thomas et al., 2011).

Previous studies showed that ferric sulfate is more cytotoxic to HGFs than aluminum chloride (Nowakowska et al., 2010) and that it inhibits the proliferation of HGFs (Liu et al., 2009). In addition, at higher concentrations, ferric sulfate significantly reduced the viability of fibroblasts derived from periodontal ligament (PDL) (Al-Haj et al., 2015). Furthermore, ferric sulfate showed marked toxicity to PDL cells compared to aluminum chloride as the time of exposure and concentration increased (Phumpatrakom et al., 2020). Although the cytotoxic effect of aluminum chloride and ferric sulfate on fibroblast cells has been evaluated (Liu et al., 2009; Nowakowska et al., 2010; Al-Haj et al., 2015; Phumpatrakom et al., 2020), the effect of these two hemostatic agents on fibroblast cell apoptosis was not investigated. Therefore, this study aims to assess and compare the apoptotic response of HGFs after exposure to aluminum chloride or ferric sulfate *in vitro.* Our null hypothesis is that aluminum chloride and/or ferric sulfate do not affect HGF apoptosis.

2. Materials and Methods

The present work was conducted after approval of the Scientific Research Ethics Committee of the Faculty of Dentistry, Minia University (Committee No. 39, Decision 689).

2.1. Culturing of the HGFs

The human gingival fibroblast cell line (HGF-1, ATCC® CRL-2014™; ATCC, Gaithersburg, Maryland, USA) was utilized and obtained from the American Type Culture Collection. The HGFs were seeded in 96-well plates (SPL Life Sciences, Korea) and incubated in complete DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), with 10% fetal bovine serum. The HGFs were incubated with 5% CO_2 in a humidified incubator at 37°C (Jouan SA, Saint-Herblain, France) for 24 hours. Thereafter, the HGFs were

rinsed twice with 250 µL of phosphate-buffered saline (PBS; Adwia Pharmaceuticals, El Sharkeya, Egypt). The HGFs were detached utilizing (0.05% EDTA and 0.25% trypsin) (Gibco) at 37 °C for 5 minutes.

2.2. Experimental Design

Two chemical hemostatic agents were utilized. Ferric sulfate (20% ferric sulfate, ViscoStat, Ultradent, USA) and aluminum chloride (25% aluminum chloride, HemoStop, JK Dental Vision, Egypt) were used. The cultured cells were allocated into three groups. The control group included the untreated HGFs that were cultured with culture medium only. The ViscoStat group included HGFs cultured with 20% ferric sulfate. The HemoStop group included HGFs cultured with 25% aluminum chloride.

2.3. Preparation of Gingival Retraction Agents

Extracts were prepared according to ISO 10993-12:2021 guidelines for biological evaluation of medical devices (International Organization for Standardization 2021). The two gingival retraction agents, ViscoStat and HemoStop were soaked in serum-free Minimum Essential Medium with Eagle buffer (MEM-E) (GIBCO, UK) at 37 °C for 7 days. After incubation, the solutions were subjected to cold centrifugation at 3000 rpm using a refrigerated centrifuge (Jouan-KI 22, France). Thereafter, filtration of the supernatants was performed utilizing filters with a pore size of 0.22 µm (Millipore, USA). These extracts were then applied to 96-well plates containing pre-cultured human gingival fibroblasts (HGFs) in two-fold serial dilutions (Fazary et al., 2021).

2.4. Assessment of viability of HGFs utilizing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

The assay was conducted at 24 hours post-treatment to evaluate cell viability. It is based on utilizing mitochondrial dehydrogenases to enzymatically reduce the yellow tetrazolium salt into purple formazan crystals in living cells.

The HGFs were plated at a density of 2×10^5 cells/mL in 96-well plates and cultured for 24 hours to allow adherence. The medium was then replaced, and 12 double-fold serial dilutions (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 4, 2, 1) were utilized in triplicate (0.1 mL/well). After 24 hours of exposure and rinsing PBS, 50 µL (of 0.5 mg/mL of MTT stock solution) was dispensed to the HGFs in every well (Vijayakumar et al., 2012).

Following a 4-hour incubation at 37°C, the elimination of the supernatant, and addition of 50 µL of dimethyl sulfoxide to every well was performed to dissolve the formazan crystals. Plates were kept for 30 minutes in the dark, and optical density was analyzed at 570 nm using a microplate reader (ELx800, Bio-Tek Instruments, USA). Cell viability percentages were calculated relative to untreated controls, and IC_{50} values were determined using nonlinear regression analysis (Vijayakumar et al., 2012).

2.5. Analysis of *Bax* and *Bcl2* Gene Expression Utilizing RT-qPCR

Expression of *Bcl2* and *Bax* genes utilizing the RT-qPCR was evaluated to compare the apoptotic effect induced by the two hemostatic agents on HGFs**.** Total RNA was isolated from the control and treated HGF groups after 24 hours of exposure using the Qiagen RNeasy Mini Kit (Germantown, MD, USA) and following the manufacturer's protocol. The concentration of the RNA was assessed utilizing a Beckman dual spectrophotometer (Beckman Instruments, Ramsey, MN, USA).

Ten nanograms of the extracted RNA were utilized to synthesize the cDNA utilizing the Applied Biosystems-High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). For amplification, the SYBR Green I PCR Master Mix was used (Thermo Fisher Scientific, Lithuania) using the Step One Real-Time PCR System (Applied Biosystems). The thermal cycles were (10 min at 95°C for initial activation, then 40 cycles of 15 s at 95°C, 20 s at 55°C, and 30 s at 72°C for amplification) (Mohamed et al., 2022). Expression levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**.** The utilized primers were **Bcl-2:** Forward: 5′-ATCGCCCTGTGGATGACTGAGT-3′, Reverse: 5′-GCCAGGAGAAATCAAACAGAGGC-3′. **Bax:**

Forward: 5′- TCAGGATGCGTCCACCAAGAAG-3′, Reverse: 5′- TGTGTCCACGGCGGCAATCATC-3′. **GAPDH:** Forward 5′-GTCTCCTCTGACTTCAACAGCG-3’, Reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

2.6. Statistical Analysis

Using IBM SPSS, Version 24 (Standard Version,NY, United States of America), data analysis was performed. To study the cytotoxic and apoptotic changes between the three experimental groups, One-way ANOVA followed by the Post hoc Tukey’s test were used. A p-value < 0.05 was regarded as statistically significant.

3. Results

3.1. Decreased Human Gingival Fibroblast Cell Viability in the Treatment Groups

After 24 hours of exposure of HGFs to ViscoStat or HemoStop, cell viability was significantly decreased in the treated HGFs in comparison to the untreated cells (p = 0.004). The ViscoStat group exhibited a significant decline in the viability percentage (p= 0.001) compared to that of the untreated control. The HemoStop group exhibited decreased HGF cell viability in comparison to the control group (p= 0.016). Non-significant difference in the cytotoxic effect on HGFs between HemoStop group and ViscoStat group (p= 0.347) (Table 1, Fig.1, 2).

Table 1: Comparison between the control, ViscoStat and HemoStop groups according to the viability of the HGFs.

	Control (1)	ViscoStat (2)	HemoStop (3)	P-value
At 24 hours	101.93 ± 1.4	58.77 ± 10.5	70.53 ± 10.8	0.004*
P-value**	1 vs 2 = 0.001	2 vs 3 = 0.347	1 vs 3 = 0.016	

*** For comparing the difference in Mean between groups, ANOVA test was used.**

**** For comparing the mean difference between groups, Post-hoc test with Bonferroni Corrections was used.**

Both ViscoStat and HemoStop also showed dose-dependent reductions in viability, affirming their concentration-dependent cytotoxic profiles (Fig. 1, 2). Moreover, the IC50 analysis derived from MTT assay data revealed that HemoStop exhibited a substantially higher IC50 value (186.41 µg/mL) compared to ViscoStat (29.18 µg/mL) (Fig.3).

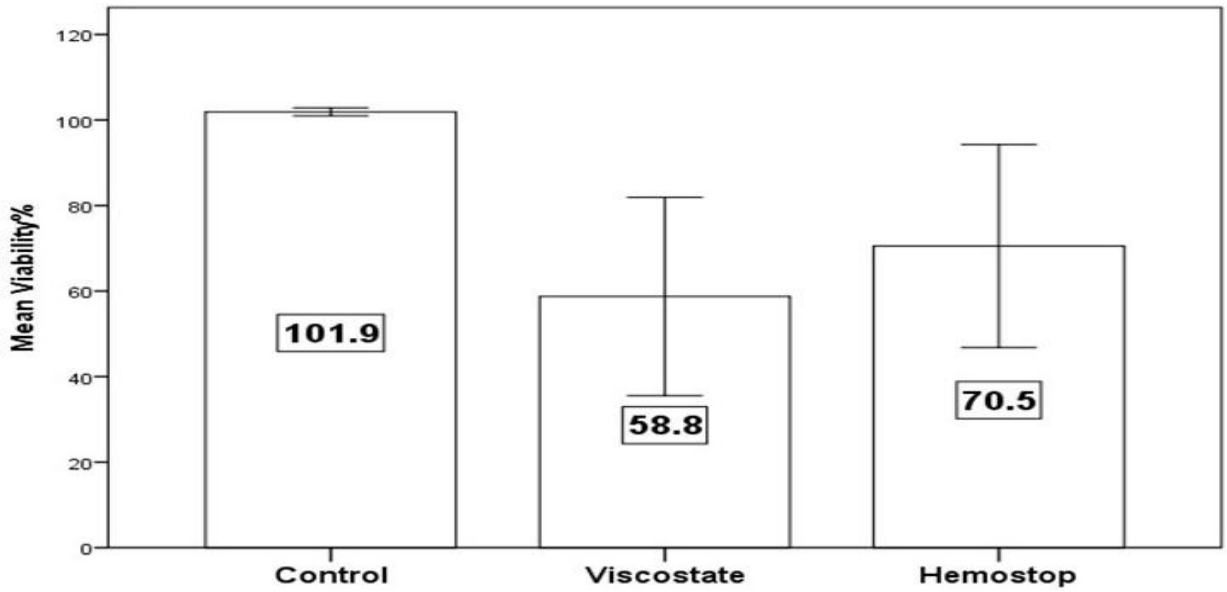

Fig 1: Effect of the treatment modalities on the Viability

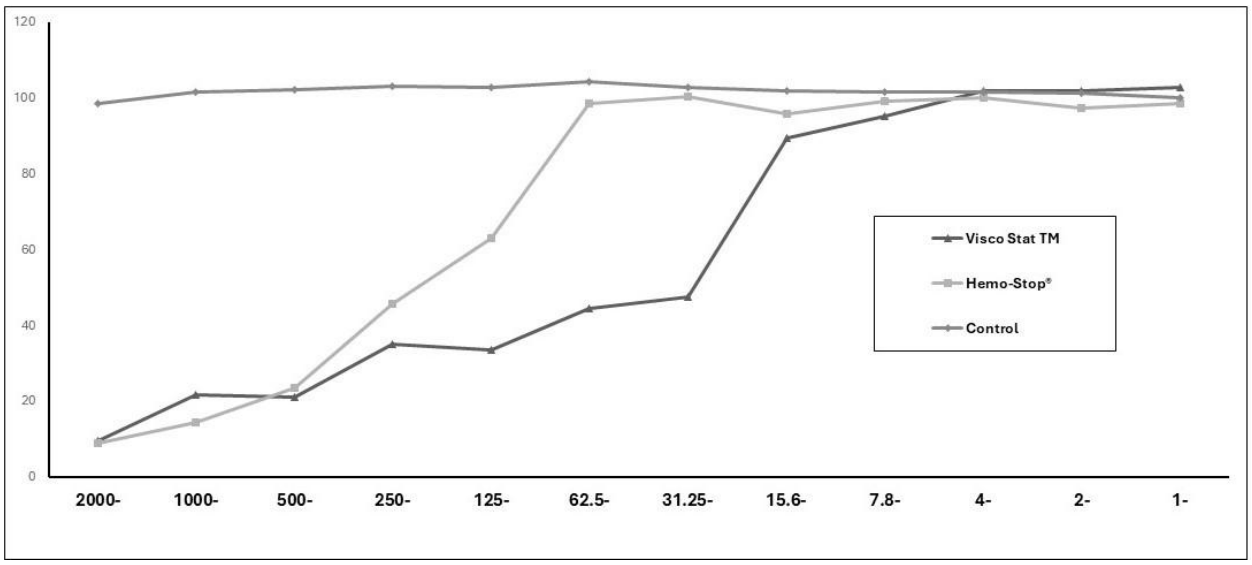

Fig. 2: Line graph illustrating human gingival fibroblast cell viability of the control, ViscoStat and HemoStop at 12 different concentrations after 24 hours of treatment

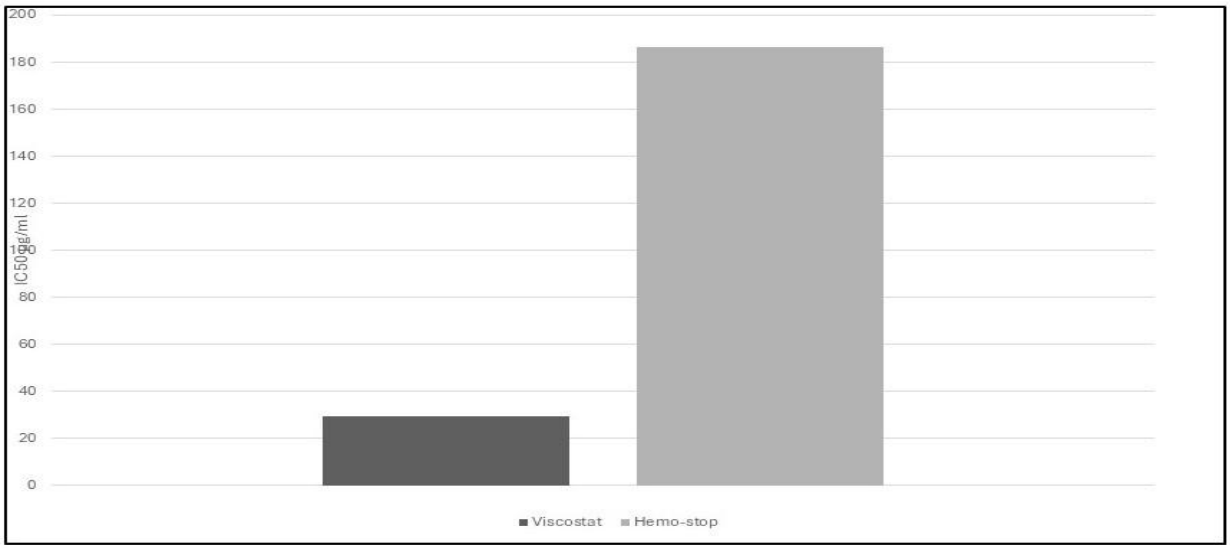

Fig. 3: Bar graph showing the IC-50 of Viscostat and HemoStop

3.2. Downregulation of *Bax* and Upregulation of *Bcl2* Gene Expression in HemoStop Treated Cells Compared to ViscoStat.

After 24 hours of exposure of HGFs to ViscoStat or HemoStop, the anti-apoptotic *Bcl2* and the pro-apoptotic gene *Bax* gene expression were assessed. The ViscoStat treated cells exhibited a 2.576-fold increase in *Bax* mRNA, which was significantly increased in comparison to the control group ($p < 0.001$). Similarly, cells treated with HemoStop demonstrated a 1.815-fold increase in *Bax* expression and was upregulated in comparison to the untreated control.

The HemoStop group depicted significantly less *Bax mRNA* in comparison to the ViscoStat group ($p = 0.001$), suggesting a more pronounced proapoptotic response induced by ViscoStat (Fig.4).

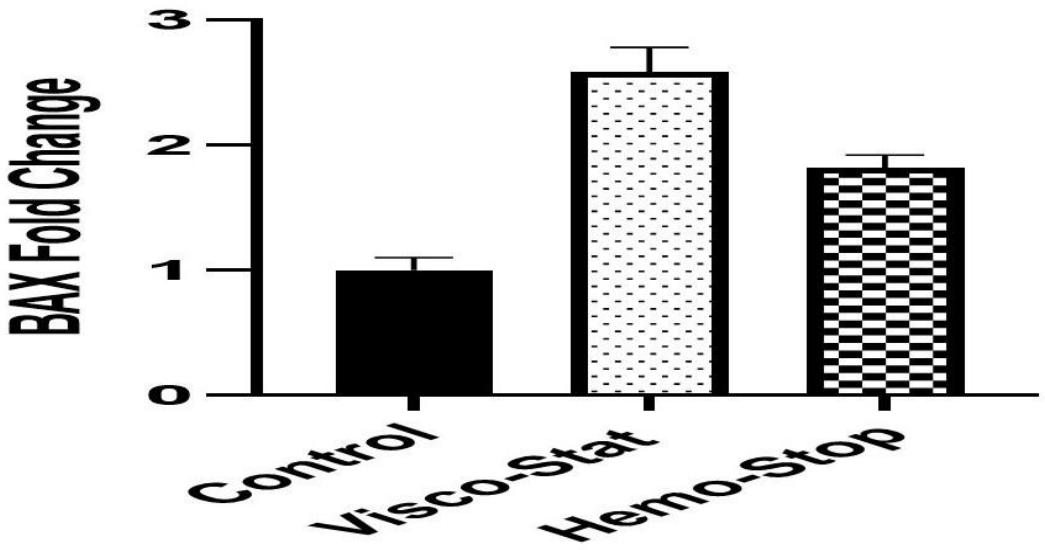

Fig. 4: Effect of ViscoStat and HemoStop treatment on BAX Fold Change in human gingival fibroblast cell line

Conversely, the expression of *Bcl2*, a critical anti-apoptotic gene, depicted a significant decrease in both treatment groups in comparison to the control. Cells exposed to ViscoStat showed a decrease in *Bcl2* expression to 0.296-fold**,** whereas the HemoStop group exhibited a moderate but still significant reduction to 0.571-fold relative to control ($p < 0.001$ for both).

Comparative analysis revealed that *Bcl2* expression was upregulated in the HemoStop group when compared to the ViscoStat group ($p = 0.004$), suggesting a lower apoptotic impact of HemoStop on HGFs compared to ViscoStat (Fig. 5).

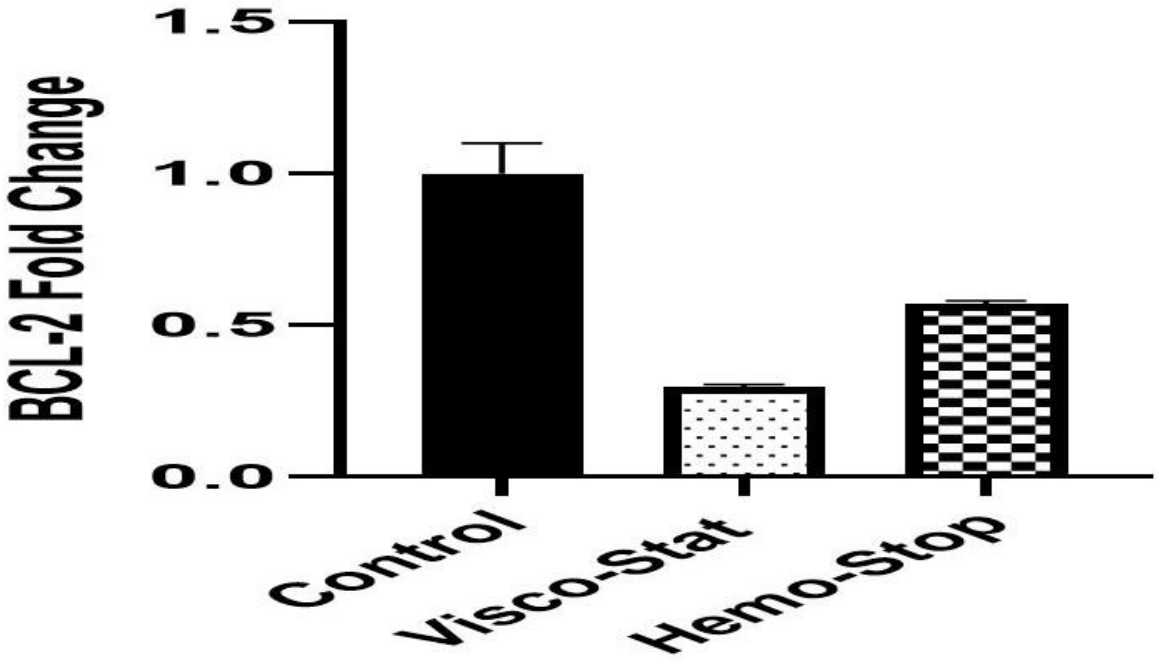

Fig. 5: Effect of ViscoStat and HemoStop treatment on BCL-2 Fold Change in human gingival fibroblast cell line.

4. Discussion

Hemostatic agents are routinely employed in dental procedures to control local bleeding and ensure a clear operative field, particularly in areas adjacent to the gingival and periodontal tissues. These agents differ markedly in their chemical composition, mechanisms of action, and biological safety. Among the most used are aluminum chloride and ferric sulfate, both of which are effective but raise concerns regarding their biocompatibility with oral tissues, especially gingival fibroblasts (Al Hamad et al., 2008).

Fibroblasts are the predominant stromal cells in gingival connective tissue, playing a vital role in maintaining tissue structure and function. They regulate homeostasis through the synthesis and remodeling of the extracellular matrix (Wielento et al., 2023), making them essential indicators of tissue response to external agents. Given this essential role, HGFs serve as an appropriate cellular model for evaluating the biological effects of dental materials, especially those intended for direct application to soft tissues (Illeperuma et al., 2012; Ahmed et al., 2025).

In this work, HGF cell line was used to evaluate the cytotoxic and apoptotic effects of two widely used hemostatic agents, aluminum chloride and ferric sulfate. The MTT viability assay and assessment of *Bcl2* anti-apoptotic and *Bax* pro-apoptotic genes by RT-qPCR were performed.

The MTT assay is widely recognized as a gold standard for cytotoxicity assessment due to its high sensitivity and adaptability for high-throughput screening. It evaluates cell viability by measuring cellular reductive capacity, primarily through the enzymatic conversion of tetrazolium salts into insoluble formazan crystals by mitochondrial dehydrogenases (Tonder et al., 2015).

Our MTT assay results showed that following 24 hours of HGFs exposure to the two hemostatic agents, cell viability in the ViscoStat and HemoStop groups was significantly decreased in comparison to the untreated group. However, a comparatively lesser reduction in viability was detected in the HemoStop group when compared to the ViscoStat group, indicating a more favorable biocompatibility profile of HemoStop. In addition, the higher IC50 values for HemoStop compared to ViscoStat suggest safety for prolonged exposure.

The data of this work are consistent with previous work (Labban et al., 2019), who found that ferric sulfate-based agents, including ViscoStat, exhibited greater cytotoxicity with longer exposure times. In contrast, aluminum chloride showed the least toxicity to HGFs among the different tested hemostatic agents. Similarly, Lodetti et al. reported dose-dependent increases in the cytotoxicity of astringent liquids to normal human gingival keratinocytes, with ferric sulfate-based products demonstrating the highest toxicity (Lodetti et al., 2004). Nowakowska et al. observed a time-and concentration-dependent cytotoxicity of several hemostatic agents to HGFs. In addition, ferric sulfate products, including ViscoStat, demonstrated the highest cytotoxic effect even after 3 minutes, while aluminum chloride agents exhibited moderate cytotoxicity. The authors explained that the key factors influencing cytotoxicity include the formulation type whether in gel or solution form and the low pH of the agents used (Nowakowska et al., 2010).

Furthermore, in agreement with our findings, Liu et al. found that ferric sulfate induced the most severe cellular degeneration and inhibition of cellular proliferation among several chemical retraction agents tested on human gingival fibroblast cells, underscoring its potential as the most cytotoxic agent among those tested (Liu et al., 2009).

In addition, our results were further supported by Al-Haj et al. who reported a slight, non-significant increase in the viability of human periodontal ligament fibroblasts at lower concentrations of ferric sulfate after 24, 48, and 72 hours. In contrast, higher concentrations of ferric sulfate resulted in a significant reduction in cell viability (Al-Haj et al., 2015). Similarly, Ahmadzadeh et al. reported that, in their *in vivo* study, ferric sulfate-impregnated retraction cords induced significant histopathological alterations in the gingival connective tissue of dogs after 3-minute and 10-minute exposure intervals (Ahmadzadeh et al., 2014).

Furthermore, Phumpatrakom et al. reported that both ferric sulfate and aluminum chloride were cytotoxic to PDL cells in a time- and concentration-dependent manner. They demonstrated that aluminum chloride exhibited moderate cytotoxicity at 1 and 5 minutes, which intensified with higher concentrations and became highly cytotoxic at 24 hours. Ferric sulfate, on the other hand, displayed a more severe profile, showing marked cytotoxicity at all exposure durations and concentrations, including significant toxicity as early as 1 minute (Phumpatrakom et al., 2020).

To investigate the genotoxic effect of the two hemostatic agents, assessment of Bcl2 and *Bax* gene expression was conducted 24 hours after treatment. *Bax* is a pro-apoptotic protein which allows mitochondria cytochrome c release, that triggers caspase activation and the subsequent degradation of cellular proteins. In contrast, the anti-apoptotic protein (*Bcl2)* functions by inhibiting Bax-mediated release of cytochrome c and suppressing caspase activation. Consequently, the balance between *Bax* and *Bcl2* expression levels is considered a key determinant in the decision between cell survival and apoptosis (Pourhajibagher et al., 2020). Therefore, the Bax/Bcl2 ratio is widely used as a measure of cell fate within the intrinsic mitochondrial apoptotic pathway (Mesole et al., 2020).

Our results revealed significant *Bax* upregulation in both treatment groups, more prominently in the ViscoStat than in the HemoStop groups. However, *Bcl2* expression was more downregulated in the ViscoStat compared to HemoStop groups, suggesting that ferric sulfate may be more potent in activating the apoptotic pathways in gingival fibroblasts. The apoptosis observed with ferric sulfate in our study could be attributed to oxidative stress as demonstrated by Li et al. who previously reported that ferric compounds could stimulate oxidative stress, release of the mitochondrial cytochrome c, and activation of caspase, confirming involvement of both intrinsic and extrinsic apoptotic pathways (Li et al., 2016). Meanwhile, studies on aluminum chloride suggested its role in disrupting cellular homeostasis by triggering mitochondrial dysfunction and promoting apoptotic signaling. However, these effects are often dose- and time-dependent and less pronounced than those caused by ferric compounds (Zhu et al., 2012; Justin-Thenmozhi et al., 2018).

5. Conclusion

In conclusion, HemoStop (25% aluminum chloride) may exhibit higher biocompatibility compared to ViscoStat (20% ferric sulfate), as demonstrated by the increased cell viability percentage and the more favorable IC_{50}. In addition, the pro-apoptotic effect of HemoStop was significantly less than that of ViscoStat and the anti-apoptotic effect was significantly higher in HemoStop than ViscoStat group suggesting less genotoxic effect of HemoStop than ViscoStat. Therefore, it is advisable to use aluminum chloride in clinical situations that require prolonged clinical contact to minimize the genotoxic effect on the local tissues. The limitation of the current work includes the deficiency of the *in vivo* investigations and the absence of other molecular studies on HGFs after exposure to the two hemostatic agents. Future *in vivo* investigations are encouraged to validate our *in vitro* observations, and further molecular investigations are required to elucidate the possible hitherto undiscovered genotoxic effect of the two chemical hemostatic agents.

Abbreviations

Bax	BCL-2-associated X protein
Bcl2	B-cell lymphoma-2
HGF	Human gingival fibroblast
HGF-1	Human gingival fibroblast cell line
MTT	3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffered saline
PDL	Periodontal ligament

CRediT Authorship Contribution Statement

Yasmien Mokhtar Hassan [(1)]: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources, Validation; Visualization; Writing - original draft; Writing - review and editing.

Hanaa Salem Moubarak [(2)]: Data curation, investigation, methodology, Supervision, Validation, Visualization, Writing - original draft, writing-review and editing

Maha El Shahawy [(3,4 *)]: Conceptualization, Data curation, Formal analysis, investigation, methodology, Supervision, Validation, Visualization, Writing - original draft, writing-review and editing

All authors have read and agreed to the published version of the manuscript.

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