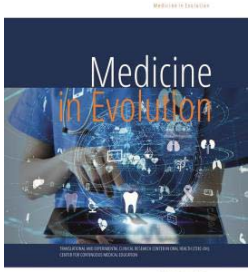
Exploring the Pharmacotoxicological Mechanisms of Botulinum Toxin on Healthy and Tumoral Oral Cells



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Abstract

This study investigates the pharmacotoxicological action mechanisms of botulinum toxin (BT) on oral cellular health, comparing its effects on healthy and tumoral cells in the oral cavity. Utilizing primary cultures of oral keratinocytes and fibroblasts, alongside oral squamous cell carcinoma (SCC) lines, the research assesses BT's cytotoxic, antiproliferative, and migration effects under controlled in vitro conditions, complemented by in ovo chorioallantoic membrane (CAM) assays to evaluate vascular irritant potential. Results indicate a clear dose-dependent cytotoxicity of BT across all cell types. Viability and proliferation rates for healthy keratinocytes and fibroblasts slightly decreased to 95% and 96% at low BT concentrations, with more pronounced effects observed in SCC cells (90% viability). At higher concentrations, viability dropped significantly to 70% for SCC cells, highlighting BT's potential for selective tumoral targeting. Cell migration assays revealed significant reductions in motility for all cell types, suggesting implications for wound healing and tumoral invasion. CAM assay outcomes demonstrated BT's minimal irritant effects at low doses, with increasing vascular irritation observed at higher concentrations. Conclusively, this study underscores the importance of concentration in BT's cellular impact, advocating for optimized therapeutic applications in dental medicine that minimize adverse effects while leveraging its antitumoral potential.

Keywords: in-vitro study, dental medicine, stomatology, botox, botulinum toxin

INTRODUCTION

Botulinum toxin (BT), a neurotoxic protein produced by Clostridium botulinum, has found extensive applications beyond its initial medical use for muscle spasms, extending into the fields of dentistry and cosmetic surgery [1]. The toxin's ability to inhibit acetylcholine release at neuromuscular junctions provides a temporary cessation of muscle activity, making it an invaluable tool for treating a variety of orofacial conditions. This includes management of conditions like hyperactive muscle disorders, which contribute to aesthetic and functional dental issues such as gingival smiles and asymmetric smile corrections [2]. Despite its broad utility, the underlying pharmacotoxicological mechanisms of BT on oral health, particularly its effects on healthy versus tumoral oral cells, remain insufficiently explored. This knowledge gap hinders the optimization of BT use in dental practices, where its potential for enhancing patient outcomes is vast.

In recent years, the application of BT in dental medicine has expanded, driven by its minimally invasive nature and reversible outcomes. This expansion includes therapeutic uses that leverage BT's muscle-relaxing properties to address conditions like bruxism, temporomandibular joint disorders, and post-surgical muscle contractions, which can significantly impact patient quality of life [3,4]. However, alongside its therapeutic benefits, there's an emerging need to thoroughly understand BT's safety profile and its long-term effects on oral cellular health. Studies have begun to investigate BT's cytotoxic effects, signaling pathways, and impact on cell proliferation and apoptosis, particularly in the context of oral mucosa and underlying cellular structures [5,6]. Such research is critical for delineating the boundaries of safe BT use and for developing protocols that minimize adverse effects while maximizing therapeutic benefits.

Moreover, the exploration into BT's effects on tumoral cells in the oral cavity presents a promising avenue for adjunctive cancer therapies. Preliminary studies have shown that BT can influence the cell cycle of tumoral cells, potentially offering a novel approach to managing oral cancer growth and proliferation [7,8]. This burgeoning area of research underscores the importance of understanding the dual nature of BT's effects on both healthy and diseased oral tissues. By delineating the mechanisms through which BT interacts with various cell types within the oral environment, researchers can unlock new therapeutic strategies and improve the safety and efficacy of BT applications in dentistry.

This study aims to elucidate the detailed pharmacotoxicological action mechanisms of botulinum toxin on oral cellular health, focusing on its differential effects on healthy and tumoral cells within the oral cavity. The primary objective is to conduct a comprehensive analysis of BT's cytotoxic effects, its influence on cell proliferation, apoptosis, and migration in both healthy oral cells and oral carcinoma cell lines. Through in vitro and in ovo studies, the project seeks to assess BT's safety profile, identify specific markers of cytotoxicity, and evaluate its potential irritant effects on blood vessels. These objectives are designed to provide a foundational understanding of BT's interactions at the cellular level, offering insights into optimizing its therapeutic use in dental medicine while minimizing potential adverse effects. By achieving these objectives, the research aims to contribute significantly to the body of knowledge in dental pharmacology, supporting the development of safer, more effective BT-based treatments for a range of dental conditions.

MATERIAL AND METHODS

The current study was designed as an in-vitro study at the “Victor Babes” University of Medicine and Pharmacy from Timisoara, in accordance with ethical regulations, and

according to the grant number 26679/09.11.2022. In our study, primary cultures of healthy oral keratinocytes and fibroblasts, along with established lines of oral squamous cell carcinoma (SCC), were utilized to assess the cytotoxic and antiproliferative effects of botulinum toxin (BT). These cells were cultured under controlled conditions (37°C, 5% CO_2) and treated with various concentrations of BT type A, reflecting its clinical application spectrum. The selection of BT concentrations and exposure times was informed by initial dose-response curves, aiming to delineate the optimal conditions that mirror the toxin's pharmacotoxicological interactions in an in vitro setting.

The response of these cell cultures to BT treatment was meticulously evaluated through a series of assays. Viability and proliferation were quantified using MTT and luminescence assays, offering a clear picture of BT's cytotoxicity across different cell types. The scratch assay method was employed to examine cell migration, providing insights into BT's influence on wound healing and tumoral cell invasiveness. Additionally, the effects of BT on apoptosis and cell cycle progression were investigated using flow cytometry and specific nuclear staining techniques. This comprehensive assay suite allowed for an in-depth evaluation of BT's cellular impacts, highlighting its potential therapeutic and adverse effects.

The chorioallantoic membrane (CAM) assay was conducted to assess BT's irritant potential on blood vessels within a living system. Fertilized chicken eggs were incubated until the CAM was suitably developed for experimental manipulation. Subsequently, BT was applied topically to the CAM, and the ensuing vascular responses, including hemorrhage, vessel lysis, and coagulation, were meticulously scored. This in ovo assay complemented our in vitro findings by presenting a complex biological context for BT's interaction with vascular structures, enriching our understanding of its pharmacotoxicological profile.

RESULTS

The data presented in Table 1 reveals a clear, dose-dependent cytotoxic effect of botulinum toxin (BT) on both healthy oral keratinocytes, fibroblasts, and oral squamous cell carcinoma (SCC) cells. With the application of BT at low concentration, a moderate reduction in cell viability and proliferation rates was observed across all cell types, indicating BT's cytotoxic potential. For healthy keratinocytes and fibroblasts, viability decreased to 95% and 96%, respectively, with a more pronounced effect seen in oral SCC cells, where viability dropped to 90%. The impact of BT was more substantial at higher concentrations, where viability fell to 85% for keratinocytes, 87% for fibroblasts, and significantly to 70% for SCC cells. Similarly, proliferation rates followed a comparable trend, underscoring the importance of concentration in BT's cytotoxicity and its potential utility in selectively targeting tumoral cells while affecting healthy cells to a lesser extent.

Table 1. Cell Viability and Proliferation after BT Treatment

Cell Type	Treatment	Viability (%)	Proliferation Rate (%)
Healthy Keratinocytes	Control	100	100
Healthy Fibroblasts	Control	100	100
Oral SCC	Control	100	100
Healthy Keratinocytes	BT Low	95	90
Healthy Fibroblasts	BT Low	96	92
Oral SCC	BT Low	90	85
Healthy Keratinocytes	BT High	85	80
Healthy Fibroblasts	BT High	87	83
Oral SCC	BT High	70	65

Table 2 demonstrates the impact of BT on the migration rates of healthy oral keratinocytes, fibroblasts, and oral SCC cells, highlighting its inhibitory effects on cell motility. Control groups showed baseline migration rates of 214 µm/24h, 158 µm/24h, and 256 µm/24h for keratinocytes, fibroblasts, and SCC cells, respectively. Post BT treatment, a significant reduction in migration rates was noted across all cell types, with keratinocytes showing a decrease to 122 µm/24h, fibroblasts to 104 µm/24h, and SCC cells to 81 µm/24h. This reduction in migration rate indicates BT's potential to impair wound healing processes and possibly inhibit tumoral cell invasion and metastasis, suggesting a dual role in therapeutic applications where modulation of cell migration is desired.

Table 2. Cell Migration Rate

Cell Type	Treatment	Migration Rate (µm/24h)
Healthy Keratinocytes	Control	214
Healthy Fibroblasts	Control	158
Oral SCC	Control	256
Healthy Keratinocytes	BT Treated	122
Healthy Fibroblasts	BT Treated	104
Oral SCC	BT Treated	81

The CAM assay results, as detailed in Table 3, provide insight into the irritant potential of BT on vascular structures at varying concentrations. At low BT concentration, there were no observable effects on hemorrhage, vessel lysis, or coagulation, indicating minimal irritant potential. However, with medium concentration, a moderate response was elicited, as evidenced by scores of 1 and 2 in hemorrhage, vessel lysis, and coagulation. The response was further amplified at high concentrations, reaching scores of 2 and 3 across all parameters. These findings suggest that while BT can be relatively safe at low concentrations, its potential to cause vascular irritation increases significantly with concentration. This highlights the importance of careful dose management in clinical applications to minimize adverse effects, especially in treatments involving vascular-rich areas such as the oral cavity.

Table 3. CAM Assay Scores for BT's Irritant Potential

Sample ID	BT Concentration	Hemorrhage Score (0-3)	Vessel Lysis Score (0-3)	Coagulation Score (0-3)
Sample 1	Low	0	0	0
Sample 2	Medium	1	1	1
Sample 3	High	2	2	2
Sample 4	Medium	1	2	2
Sample 5	High	3	3	3

DISCUSSIONS

The findings from this study offer significant insights into the pharmacotoxicological mechanisms of botulinum toxin (BT) on oral cellular health, emphasizing its differential effects on healthy and tumoral cells within the oral cavity. The dose-dependent cytotoxicity of BT underlines the toxin's potential for therapeutic application, particularly in selectively targeting tumoral cells. A noteworthy observation was the pronounced reduction in viability and proliferation rates in oral squamous cell carcinoma cells at higher BT concentrations, suggesting BT's utility in managing oral carcinomas. This specificity could be leveraged to minimize collateral damage to healthy oral cells, a critical consideration in developing safer, more effective BT-based treatments for oral conditions.

The reduction in cell migration rates post-BT treatment highlights another dimension of BT's therapeutic potential. The significant decrease in migration rates across all cell types, especially in oral SCC cells, suggests BT's role in not only impairing wound healing processes but also in potentially inhibiting tumor invasion and metastasis. This dual functionality points towards the necessity of a balanced approach in BT's clinical applications, where its benefits in reducing tumoral cell motility must be weighed against potential delays in normal wound healing.

CAM assay findings introduce critical considerations regarding BT's irritant potential on vascular structures. The escalation in hemorrhage, vessel lysis, and coagulation scores with increasing BT concentrations underscores the importance of careful dose management to mitigate vascular irritation. This aspect is particularly pertinent in dental applications, where the oral cavity's vascular richness demands a nuanced understanding of BT's effects to avoid adverse outcomes. These results advocate for a cautious optimization of BT's concentration in therapeutic interventions, ensuring efficacy while minimizing potential harm to vascular health.

Over the past twenty years, research into the use of Botulinum neurotoxins for cancer treatment, particularly for shrinking tumors and inducing apoptosis in cancer cells, has significantly expanded. Early investigations, such as the one by Huang et al. in 1998 [9], focused on the effects of BoNT/A on insulin secretion by insulin-secreting HIT-T15 cells, a type of endocrine tumor cell. These studies, employing animal models, human subjects, and in vitro applications of BoNTs on various cancer cell lines, including those from neuroblastoma, breast, prostate, colorectal, and pancreatic cancers, have shown promising results for BoNT/A's potential in cancer therapy. Huang et al.'s work notably demonstrated the ability to regulate insulin secretion through transient transfection of BoNT/A, setting a foundational basis for further exploration of BoNT/A in treating endocrine tumors.

Karsenty et al.'s 2009 [10] study further explored BoNT/A's effects, particularly on prostate cancer cell lines PC-3 and LNCaP, noting a significant reduction in LNCaP cell proliferation and an increase in apoptosis in a dose-dependent manner, without affecting PC-3 cells. This difference was attributed to the presence of the SV2 receptor, with LNCaP cells showing a higher expression ratio. The study highlighted ONA's (OnabotulinumtoxinA) potential to slow growth rate and PSA progression, marking a significant step forward in understanding BoNT/A's therapeutic mechanisms in cancer.

Proietti et al. in 2012 [11] expanded this line of inquiry by examining the effects of different doses of IncoA (IncobotulinumtoxinA) on prostate cancer cells, observing a reduction in cell growth for both LNCap and PC-3 lines. Their work underscored the role of the SV2 receptor and introduced the cPLA2-α expression as a potential marker for BoNT/A's effectiveness. In contrast, Bandala et al.'s study in 2015 [12] focused on breast cancer cells, demonstrating BoNT/A's cytotoxic effects on the T47D cell line and its impact on SV2 receptor expression across various breast cancer cell lines. These studies not only contributed to our understanding of BoNT/A's apoptotic mechanisms but also proposed the SV2 receptor as a potential molecular marker for breast cancer, suggesting innovative approaches for BoNT/A utilization in cancer therapy.

This study, while providing valuable insights into the pharmacotoxicological effects of botulinum toxin (BT) on oral health, has limitations inherent to in vitro and in ovo research models. The direct applicability of these findings to clinical scenarios is constrained by the simplified experimental conditions, which may not fully capture the complex interactions within the human oral cavity or the systemic effects of BT. Additionally, the use of established cell lines, while offering controlled conditions for assessing BT's effects, may not accurately reflect the diversity of cellular responses in different individual's oral tissues. The reliance on these models necessitates cautious interpretation of results and underscores the need for

subsequent clinical studies to validate the therapeutic potential and safety profile of BT in dental applications.

CONCLUSIONS

In conclusion, this study contributes valuable data to the body of knowledge in dental pharmacology, delineating BT's effects on oral health at the cellular level. By identifying specific markers of cytotoxicity and evaluating its impact on cell proliferation, apoptosis, migration, and vascular irritation, the research paves the way for optimizing BT's therapeutic use in dentistry. The insights gleaned from this study underscore the potential of BT as a versatile therapeutic agent in oral medicine, capable of addressing a range of dental conditions with appropriate dose management and application strategies. Future research should further explore BT's long-term effects and its integration into comprehensive treatment modalities, ensuring the development of safe and effective BT-based therapies for oral health conditions.

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