Therapeutic effect of exosomes derived from bone marrow mesenchymal stem cells on the lingual papillae mucositis induced by 5-Fluorouracil in Albino rats

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Abstract:

Fluorouracil (5-FU) is one of the prevalent anti-cancer drugs that is commonly used into chemotherapeutic modalities to treat head and neck solid cancers. However, various studies have reported that 5-FU has the ability to generate toxicity in different tissues causing oral mucositis. Using exosomes derived from stem cells is gaining a huge attention for therapies as it may have certain advantages over adult stem cell-based therapies. This study aimed to evaluate the therapeutic effect of bone marrow stem cells derived exosomes on the cytotoxicity induced by 5-Fluorouracil on the lingual papillae of the albino rats. Thirty male Albino rats weighing between 150 - 200 grams were equally and randomly divided into 3 groups as follows: Group I (control group): containing 10 rats, received saline daily via oral gavage for 14 days. Group II (5-FU group): containing 10 rats, received a single IP injection of 5-FU® drug (50 mg/kg) each. Group III (5-FU + Exosomes group): containing 10 rats, the rats was treated as group II, then injected by a single injection through the tail vein of exosomes on day 2 (100 µg/kg/dose suspended in 0.2 ml PBS). Rats of each group were euthanized after 14 days, and tongue specimens were collected for histological, ultrastructural and immunohistochemical evaluation. In 5-FU treated group, there were marked degenerative changes of the filiform such as areas of keratin separation and hyperkeratinization and the fungiform papillae showed atrophy and deformed taste buds. While the Exosomes treated group showed marked improvement in the histological, ultrastructural and immunohistochemical results. Exosomes derived from bone marrow mesenchymal stem cells exerted an obvious therapeutic effect against the induced mucositis caused by the cytotoxic effect of 5-flourouracil represented by improved histological, ultrastructural and immunohistochemical results.

Keywords: 5-Fluorouracil, BAX, Exosomes, Mesenchymal Stem Cells derived Exosomes, Oral Mucositis.

1- Introduction

5-Fluorouracil (5-FU) is an antimetabolite drug which is frequently utilized as an affective chemotherapeutic agent. It was discovered that this drug is effective against a widespread diversity of solid tumors and is considered by the World Health Organization (WHO) as one of the most effective drugs used in cancer treatment. Until now, 5-FU continues to be the source of treatment for cancers of the digestive tract, colorectal cancer, as well as cancers arising in other organs (1).

5-FU has a multifaceted cytotoxic activity that involves thymidylate synthase inhibition and the integration of its metabolites into RNA and DNA. Since thymidine is essential for both DNA replication and repair, DNA replication arrest is caused by a huge increase in dUTP levels and unusually low dTTP levels when thymidylate synthase is inhibited. Additionally, 5-FU may be integrated into the structure of RNA, impairing its ability to operate normally. Consequently, the DNA and RNA synthesis disruption caused by 5-FU results in the suppression of cell division which directs the cell towards apoptosis (2).

Oral mucositis is a common distressing adverse effect of 5-FU. Several methods of intervention that have been explored to overcome the oral mucositis induced by 5-fluorouracil (5-FU) chemotherapy such as cryotherapy, low-level laser therapy and keratinocyte growth factor. However, several limitations have been found concerning the previous modalities (3).

Mesenchymal stem cells (MSCs) have been vastly utilized in numerous inflammatory diseases as they have several characteristics such as directed differentiation, antioxidant, anti-apoptosis and promotion of cell regeneration. Mesenchymal stem cells are believed to possess their therapeutic effect through the release of various factors such as cytokines, growth factors, and exosomes. Exosomes are nanosized (30–150 nm in diameter), circularly secreted external membrane vesicles that are released by cells and transport several types of proteins, RNA, metabolites, and lipid membrane components to adjacent and distant cells. Exosomes super passes the benefits of MSCs by having the benefits of targeted administration, minor immunogenicity, and high repairability (4).

Exosomes derived from MSCs main function is cell-to-cell communication as they transport nucleic acids such as mRNAs, miRNAs and lncRNAs, that can modulate target genes. The promising role of MSC-exosomes was confirmed by a recent study as in wound healing and alleviation of scars through re-epithelization, angiogenesis, and regulation of collagen remodeling (5).

Exosomes therapeutic potential is gaining an increased attention based on several researches within the orofacial discipline as they have a promising potential in different extents, such as the acceleration of wound healing in tongue defects as well as enhancing wound healing with minimal scar formation (5, 6). Therefore, the current study sought to assess the efficacy of bone marrow MSC-derived exosomes in alleviating the adverse effects of 5-FU on the lingual papillae of Albino rats. The null hypothesis of the current research was that exosomes wouldn't have a significant therapeutic effect on the 5-FU induced lingual papillae mucositis.

2- Materials and Methods:

2.1 Study Setting and Sample Selection:

The experiment was performed following the ethical guidelines of animals experimentation and was evaluated by the Faculty of Dentistry's research ethics committee (REC), Suez Canal University, Egypt (475/2022).

The sample size for this study was calculated using the following equation:

Total sample size N = $\frac{(1.96)^2 x (5.58)^2}{(2)^2}$ **= 29.903≈ 30 samples**

2.2 Study Procedures:

2.2.1 Exosomes Preparation:

A- BMMSCs Isolation and Culture: Bone marrow cells were gained from the tibia of albino male rats. Phosphate-buffered saline (PBS) was used to wash the cells. The cells flushed were put on 15 ml of Ficoll-Paque (Gibco Invitrogen, Grand Island, NY) and centrifuged at 400 g for 35 minutes. Cells at the interface were aspirated and washed twice in sterile PBS and centrifuged for 10 min at 200 x g 5° C. The isolated BMMSCs were cultured in a DMEM medium supplemented with 10% FBS and penicillin. Cells of the third passage (P3) were used in the study.

B- Identification of BMMSCs: Morphology of BMMSCs under TEM and flow cytometric analysis for the cluster of differentiation's positivity 73, 90, and 105 and negativity of CD 34 and 45 were assessed.

C- Exosomes Derived from BM-MSCs preparation: The exosomes were obtained using a cell supernatant exosome isolation kit (Thermo Fisher Scientific). They were then suspended in PBS and stored at -20 °C for future usage. Exosomes were identified using transmission electron microscopy and flow cytometry to detect CD 63 and 81 positivity as well as fluorescent dye labelling.

2.3. Animals Grouping and Treatment Protocol:

Thirty male albino rats each weighs between 150 - 200 grams were housed in cages in the animal house of Faculty of Dentistry, Suez Canal University. Rats were kept in a 12h/12h dark and light cycle with free food and water adlibitum access. Rats were equally and randomly divided into three groups, each consisting of 10 albino rats as follows:

1. **Group I (control group)**: Rats received saline daily via oral gavage for 14 days.
2. **Group II (5-FU group)**: Rats were injected by a single intraperitoneal (IP) injection of 5-FU® drug (50 mg/kg) each (7). 5-FU ® Utoral 250 mg/5 ml solution Vial for intravenous (IV) infusion was purchased from Hikma Specialized Pharmaceuticals, in the Second Industrial Zone, 6th of October, Giza, Egypt.
3. **Group III** (**Exosomes group**): Rats were treated as group II, then on day 2, a single injection of exosomes was given to the rats through their tail vein (100 µg/kg/dose suspended in 0.2 ml PBS) (8)**.**

After the experiment period, the animals were sacrificed with an extra dose of anesthesia, and the tongue from each rat were excised. The tissues were prepared for histological, ultrastructural and immunohistochemical evaluations.

2.4 Methods of Evaluation:

2.4.1 Histological Evaluation: After the fixation of the gland specimens in 10% neutral buffered formalin, the specimens were dehydrated by their immersion in successive ascending concentrations of ethanol (60%, 70%, and 95%). Then, they were infiltrated with molten paraffin wax (55%) and were embedded after that in paraffin wax blocks. The blocks were cut with a microtome to obtain 4-5 µm thickness sections which were mounted to glass slides and then stained with hematoxylin and eosin. After staining, the slides were examined under a light microscope.

2.4.2 Ultrastructural evaluation: Tongue specimens were fixed in 4% phosphate-buffered glutaraldehyde (0.1 mol/L, pH 7.4), then they were post-fixed in 1% phosphate-buffered osmium tetroxide. Dehydration of the specimens was then done by placing them in successive dilutions of ethanol and placed into amyl acetate. Samples after that got dried with liquid CO2 and were gold sputtered with a thin coat of gold particles. The tongues' dorsal surfaces were examined under a (Thermo Fisher USA Quattro S field emission gun) connected with EDAX at the nanotechnology research center (N.T.R.C) at the British University in Egypt.

2.4.3. Immunohistochemical Evaluation: Other sections of 5 microns thickness were processed and stained using rabbit polyclonal mouse antibody to Bcl-2 Associated X protein (BAX) (Santa Cruz Biotechnology, Cat. No. sc- 526) which was used for apoptotic assessment. BAX protein activation results in the increase of permeability of the mitochondrial outer membrane, release of cytochrome c, and activation of caspases with brown cytoplasmic and cell membranous expression. Slides were examined with ZEISS primo star light microscopy photographed by Tucsen IS 1000 10.0 MP camera. The mean area percentage expression of the markers was measured using ImageJ software (9).

2.4.4 Statistical Analysis

The statistical analysis were administered using GraphPad Prism software version 6.0. The results are reported as the mean value and the standard deviation. The statistical analysis was performed using One-way ANOVA, followed by a post-hoc Tukey's test. A significance level of p-value <0.05 was employed to determine the significance of all results. The experiments were replicated in triplicates, each with 3 to 6 repetitions.

3- Results:

3.1 Isolation, identification, and characterization of BMMSCs

BMMSCs were isolated and identified by their spindle-fusiform shape and formation of colonies (Fig. 1A). Moreover, characterization by flow cytometry analysis revealed that BMMSCs have a positive expression for CD73, CD90, and CD105 surface markers and a negative expression for CD34 and CD45 (Fig. 1B).

3.2. Characterization of exosomes:

1.Transmission electron microscopy (TEM) was used to characterize exosomes derived from BMMSCs. These exosomes were characterized by their consistent size range of 30-100 nm and spherical shape (Figure 1C).

2. Furthermore, the positive expression of specific markers CD63 and CD81 was confirmed using flow cytometry analysis (Figure 1D).

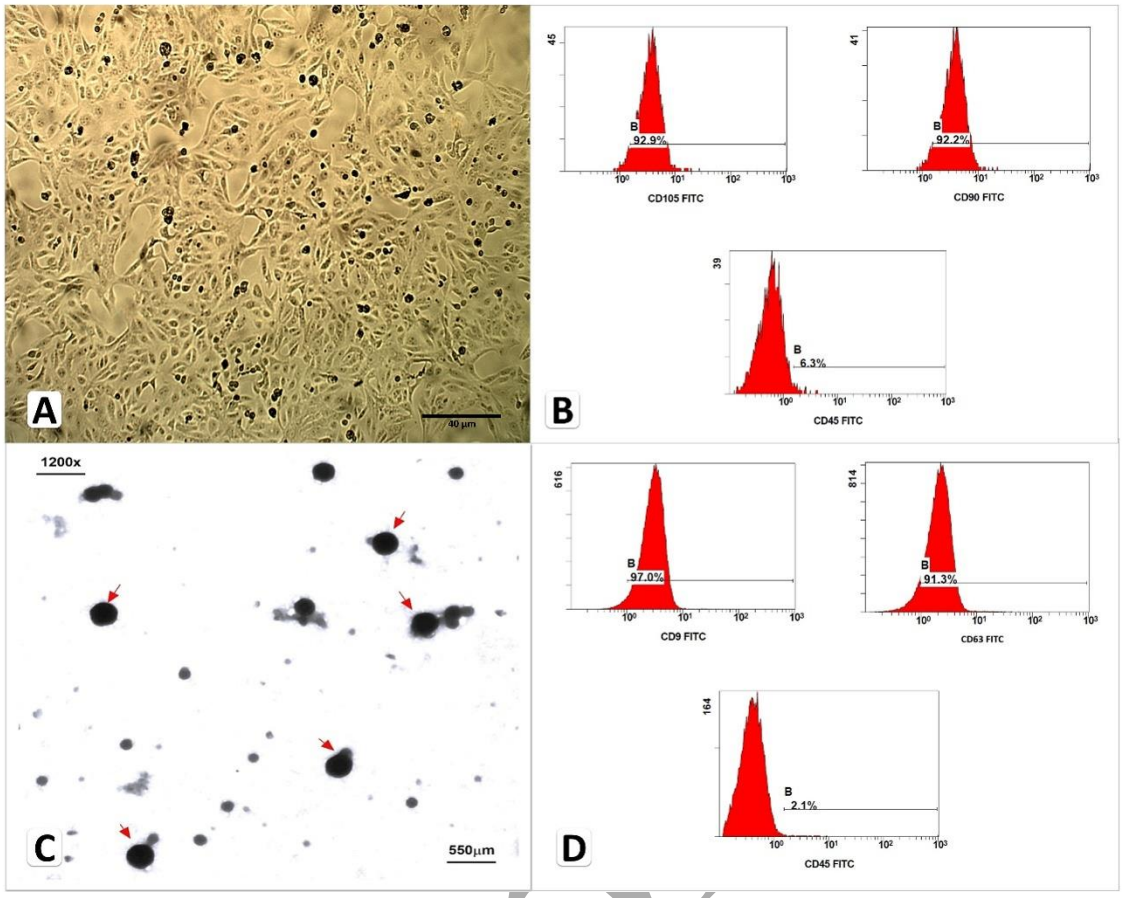

Figure 1. (A) Spindle-shaped cells identifying the BMMSCs by inverted microscope. (B) Characterization of BMMSCs by flowcytometry analysis revealing positive expression of CD73, 90, and 105 as well as negative expression of CD34 and 45. (C) Exosomes derived BMMSCs characterization of by TEM. (D) Characterization of exosomes by flowcytometry analysis showing positive expression of CD63 and 81.

3.2. Histological results:

I. Group I (Control group): Histological examination of the dorsal surfaces group I rats revealed normal histological picture. The dorsal surface of the tongue was lined by keratinized stratified squamous epithelium. Filiform papillae were the most numerous with a conical shape and tapering tips (Fig. 2.A). Fungiform papillae were few, short and scattered among the filiform ones. The apices of the fungiform were broad with a taste bud on their top with a distinctive taste pore. (Fig. 2.B).

II. Group II (5-FU group): The histological examination of the dorsal surfaces of rats treated with 5-FU showed severe atrophic and degenerative changes. The filiform papillae exhibited areas of keratin separation and hyper keratinization, with lining epithelial cells displaying vacuolated cytoplasm and pyknotic nuclei across several layers (Fig.2.C). The fungiform papillae were deformed in their epithelium and taste buds (Fig.2.D).

III. Group III (Exosomes group): The examined tongue of rats that received exosomes derived from BMMSCs showed marked improvement of the histological structure. Filiform papillae exhibited partial improvement, showing tapering ends with few short blunt-ended papillae (Fig.2.E). The fungiform papillae looked nearly normal, with well-defined taste buds and minimal signs of deformation (Fig.2.F).

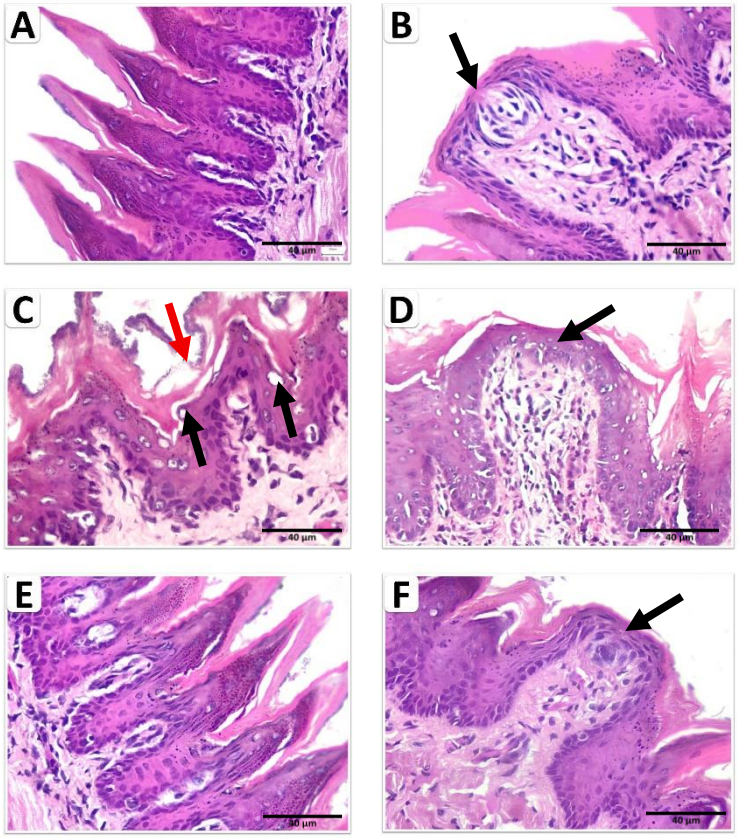

Figure 2. A. Dorsal surface of control group showing the filiform papillae. **B.** Fungi form papillae of control group with a taste bud with a well-defined taste pore (arrow). **C.** Dorsal surface of the 5-FU group showing deformed filiform papillae with areas of keratin separation (red arrow). Apoptotic cell figures of the epithelium were seen (black arrows). **D.** Fungiform papilla of 5-FU group showed atrophied and deformed shape with obliteration of taste bud (arrow). **E.** Filiform papilla of Exosomes group showing almost normal shape with blunt ends. **F.** Fungiform papilla of exosomes group showing an almost normal shape with a taste bud (arrow). **(H&E. original magnification 400)**

3.3. Ultrastructural results:

I. Group I (Control group): The dorsal surface of the control group demonstrated numerous filiform papillae, which were simple conical structures found particularly on the tip and lateral edges of the dorsal surface of the tongue (Fig.3.A). Interspersed among these were fungiform papillae, characterized by their dome- or mushroom-like shape and a central depression known as the taste pore (Fig.3.B).

II. Group II (5-FU group): The dorsal surface of 5-FU group which received 5- FU revealed filiform papillae with blunted tips, apparent decrease in thickness and number. They were arranged in an irregular pattern with a random distribution and showed surface desquamation (Fig.3.C). The fungiform papillae lost the characteristic appearance of the mushroom shape and exhibited ill-defined taste pores, sometimes absence of the taste pore, and desquamation of superficial cells were seen (Fig.3.D).

III. Group III (Exosomes group): The dorsal surface of group III animals treated with exosomes showed marked improvement. Almost normal filiform papillae were seen amongst some papillae showing signs of disorientation with rough keratinized base (Fig.3.E). Fungiform papillae were almost normal with a well-defined taste pore (Fig.3.F).

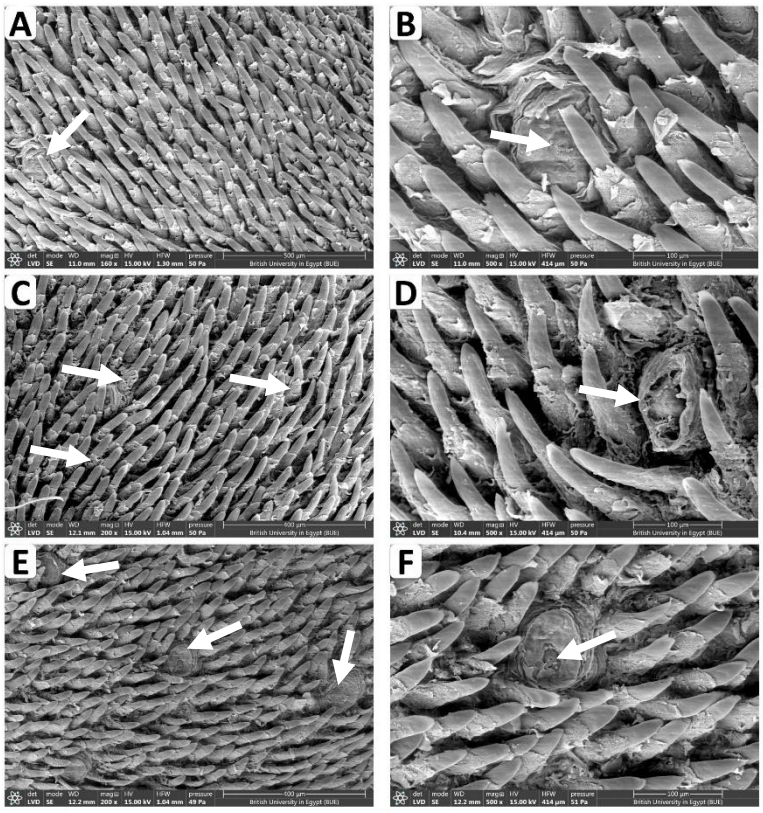

Figure 3. A. Scanning electron micrograph of the control rat's dorsal surface showing sharp and parallel rows of conical filiform papillae & a fungiform papilla in between (arrow). **B.** A higher magnification of the previous figure showing fungiform papillae of control group with a taste bud and a well-defined taste pore (arrow). **C.** 5-FU group showing randomly distributed and irregularly arranged filiform papillae with more or less blunt apex with an apparent surface desquamation as well as marked aggregation of keratin (arrows). **D.** Dorsal surface of the 5-FU group showed deformed fungiform papilla with an ill-defined taste pore (arrow). **E.** Scanning electron micrograph of the group III rat tongue showing an almost filiform papillae with few disoriented papillae. Numerous almost normal fungiform papillae were seen in between the filiform ones (arrows). **F.** Fungiform papilla of exosomes group showing an almost normal shape with a taste bud and a well-defined taste pore (arrow).

3.4. Immunohistochemical results:

BAX immunostaining was used for the evaluation of apoptosis through the assessment of its staining reaction in the tissues examined from the three groups. **Group I (Control group)**, BAX cytoplasmic staining showed a mild reaction restricted to the superficial layers of the lingual epithelium (Fig. 4.A). While in **Group II (5-FU)** rats, BAX expression increased and expanded through the entire epithelial thickness of the tongues (Fig. 4.B). In **Group III (Exosomes)** rats, BAX expression showed a mild to moderate cytoplasmic staining reaction through the lingual epithelial thickness of the tongues (Fig. 4.C).

3.5. Statistical results:

Mean area percent of Bax immunoexpression:

The highest mean area percent of BAX immunoexpression was recorded in the 5- FU group, followed by exosomes group, while the lowest mean area percent was observed in group I control. ANOVA results showed a statistically significant difference between groups ($p<0.0001$) (Fig. 4.D).

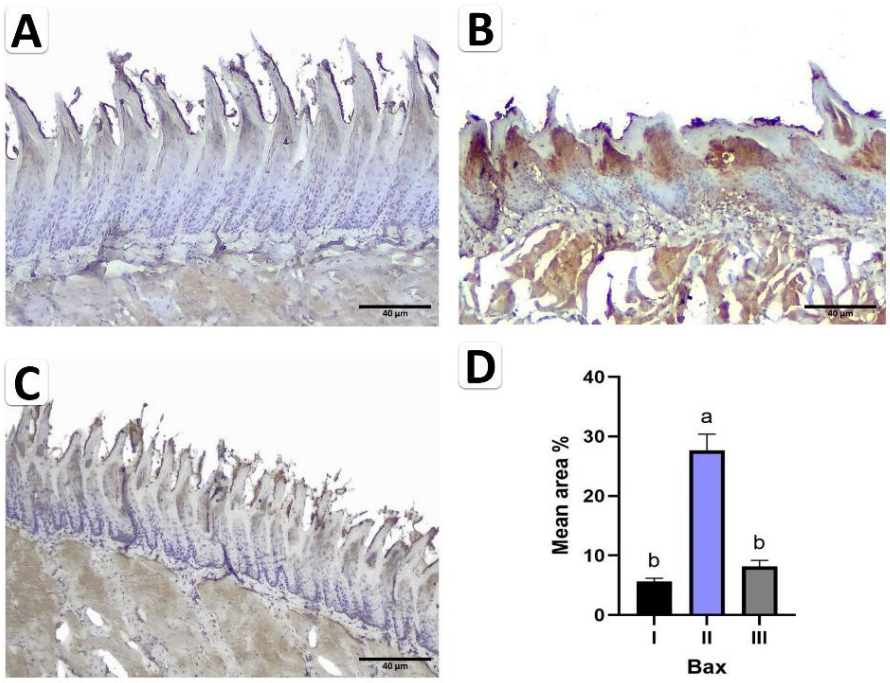

Figure 4: (A) A photomicrograph of the dorsal surface of the tongue of the control group Bax immunostaining showed mild cytoplasmic staining reaction of the superficial layers of the lingual epithelium, **(B)** While 5-FU group showed strong cytoplasmic staining reaction. **(C)** Exosomes group showed moderate cytoplasmic staining reaction through the lingual epithelial thickness. **(D)** Graph showing mean, standard deviation, and multiple comparison test of Bax mean area % immunoexpression. Different letters indicate significance.

4- Discussion

Emerging research has pointed to the therapeutic potential of exosomes derived from mesenchymal stem cells (MSCs-EXOs) in mitigating tissue damage caused by inflammation and cytotoxic agents. Given their immunomodulatory and regenerative properties, bone marrow MSC-derived exosomes hold promise as a novel treatment for 5-FU-induced toxicity (10). Accordingly, this study sought to evaluate the efficacy of bone marrow MSC-derived exosomes in alleviating the adverse effects of 5-FU on the lingual papillae of Albino rats. The tongue was selected as the focus of this study due to its critical functions in mastication, speech, and general health monitoring. The cytotoxic impacts of 5-FU on the tongue serve as a reliable model for studying OM, as the tongue is highly vascularized and metabolically active, making it susceptible to chemotherapeutic damage (11). Exosomes dose (100 µg/kg/dose suspended in 0.2 ml PBS) through the tail vein was the dose of choice in accordance with Ebrahim et al. (2018) (8) who administered the same single dose to rats examining the potential role of MSC-derived exosomes for enhancement of autophagy activity and their effect on diabetic nephropathy. Moreover, exosomes derived from mesenchymal stem cells have been proven to promote oral mucositis healing as mentioned by Gao et al. (2022) (12).

In this study, tongues of 5-FU–treated rats (Group II) showed marked degenerative histopathology in the filiform and fungiform papillae. These results were consistent with Elmansy et al. (2020) (13), where they reported similar 5-FU–induced changes in rat tongue mucosa. They noted that filiform papillae, due to their high metabolic activity, reflect overall health; thus, drug toxicity can lead to papillary atrophy. They further attributed chemotherapy-related alterations to disrupted protein synthesis and increased apoptosis.

Moreover, the degenerative histopathological effects of 5-FU on the fungiform in the current study were similar to those found by Arafat et al. (2021) (14) who studied the effect of Irinotecan, another chemotherapeutic drug similar to 5-FU on the tongue mucosa and revealed the atrophy of the fungiform papillae and showed ill-distinguished taste buds. In the same instance, a significant thickening of the keratin layer was detected, this was explained to be due to the high sensitivity of the taste buds to chemotherapy.

The ultrastructural results of the current study of the tongues of group II rats showed that the filiform papillae had blunted tips, irregularly arranged as well as showing surface desquamation. The fungiform papillae also lost their characteristic mushroom shape and revealed ill-defined taste pores which were similar to those found by Shalaby et.al (2021) (15) when they investigated the possible adverse effect of 5-FU on the mucosa of the tongue. The injury to the oral mucosal tissues due to the use 5 - FU was believed to be due to the oxidative stress production and the release of reactive oxygen species (ROS), that stimulates numerous cellular signals which initiates mucosal damage as stated by Chen et al. (2007) (16)**.**

These results were further confirmed by a previous study which declared that 5-FU injection induces a potential oxidative stress which leads to cell death. When ROS are released, pro-inflammatory cytokines are subsequently produced, which causes tissue damage and apoptosis. NF-κB is a crucial component of this process and is involved in the mucositis apoptotic pathway (17).

The immunohistochemical results using Bcl2 Associated X protein (BAX) which was used as a pro-apoptotic marker to assess the apoptotic reaction in the tongues mucosa of group II rats where confirming the previously mentioned results were an increased Bax expression that extended all over lingual epithelial thickness of the tongues of group II rats was observed.

BAX increased with 5-FU exposure seen by Mahran et al. (2024) (18) as a marker of hepatotoxicity and, across cancers, higher BAX generally tracks stronger chemotherapy response, while BAX silencing confers 5-FU resistance. Mechanistically, raising the BAX/Bcl-2 ratio stimulates the mitochondrial outer-membrane permeability, cytochrome-c release, loss of mitochondrial potential, activation of caspase and eventually apoptosis (13).

In the ongoing study, the examined tongue of rats that received exosomes derived from bone marrow stem cells (group III) presented noticeable improvement in the number and overall features of the filiform and fungiform papillae with minimal signs of deformations in the taste buds. Li et al. (2025) (19) supported these results were found that the adipose mesenchymal stem cell-derived exosomes (ADMSCs-Exos) alleviated the inflammation of oral mucosal epithelium of irradiated mice and concluded that adipose mesenchymal stem cell-derived exosomes promoted the

proliferation of oral mucosal epithelial cells and liberated the oral mucosal inflammation in mice having radiation-induced oral mucositis.

The mechanisms of oral mucositis induced by either radiotherapy or chemotherapy was explained by a review study where the authors concluded that NF-κB signal transduction pathway and pro-inflammatory cytokines have been reported to contribute to the pathogenesis of OM (20). Consequently, targeting the inhibition of NF-κB and its related pro-inflammatory cytokines has emerged as a primary focus for the treatment of OM.

Considering the similarity in the mechanisms of OM induced by cancer therapy as 5-FU, the results of the current study were consistent with the previously mentioned studies, as it has been reported that the re-epithelialization have been accelerated in the MSCs-Exos treated mice. Exosomes likely act by attenuating NF-κB signaling and downstream cytokines (TNF-α, IL-1β, IL-6), aligning with MSC-exosome studies in OM and mucositis biology (21).

Immunohistochemistry showed a significant reduction in BAX expression in the exosome-treated group (Group III) versus 5-FU alone (Group II; $p < 0.05$), indicating less apoptosis in the tongue mucosa. This aligns with Zhang et al. (2022) (22), where they found that adipose-derived MSC exosomes lower ROS and inflammation, accelerating wound healing.

The results of this study underscore the potential of bone marrow MSC-derived exosomes as a therapeutic intervention for 5-FU-induced OM. By reducing inflammation, oxidative stress, and apoptosis, exosomes address the multifactorial pathogenesis of OM and facilitate tissue repair. These findings pave the way for further exploration of MSC-derived exosomes in clinical applications, particularly for managing the adverse effects caused by chemotherapy.

The current investigation proved that exosomes derived from BMMSCs exerted an obvious therapeutic effect against the induced mucositis caused by the cytotoxic effect of 5-Flourouracil.

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Authors' contribution

Study concept and design: S.M.S, E.F.M.

Analysis and interpretation of data: E.M.H, M.M.A.

Investigation: S.M.S, M.M.A, R.A.G.

Drafting of the manuscript: S.M.S.

Critical revision of the manuscript for important intellectual content: E.F.M, E.M.H.

Study supervision: E.M.H, M.M.A, R.A.G, E.F.M.

Ethics: The research was granted ethical approval (475/2022) under the guidelines of animal experimentation and reviewed by the Faculty of Dentistry's research ethics committee (REC), Suez Canal University, Egypt.

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Data Availability: The data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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