EGYPTIAN DENTAL JOURNAL

VOL. 65, 1217:1228, APRIL, 2019

I.S.S.N 0070-9484

ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY

www.eda-egypt.org • Codex : 85/1904

DECREASED VEGF AND CYCLIN D1 GENES EXPRESSION ENHANCES CHEMOSENSITIVITY OF HUMAN SQUAMOUS CELL CARCINOMA CELLS TO 5-FLUOROURACIL AND/OR MESENCHYMAL STEM CELLS-DERIVED MICROVESICLES

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ABSTRACT

Background: Among the most common oral cancers is oral squamous cell carcinoma which has high rates of mortality. 5-flurouracil (5-FU) is a widely used chemotherapeutic agent for treatment of head and neck cancers. However, its use has many limitations due to its well- known side effects. Thus, in the current study we aimed to investigate the chemosensitivity of squamous cell carcinoma cells to 5-FU and/or mesenchymal stem cells-derived microvesicles via H&E histopathological examination, in addition to detection of vascular endothelial growth factor (VEGF) and cyclin D1 (CD1) genes' expression through quantitative RT-PCR analysis.

Material & Methods: Human squamous cell carcinoma cell line (SCC152) was treated by 5-FU or Microvesicles or their combination for 24 and 48 hours. Histopathological examination through H&E stain as well as quantitative RT-PCR analysis, for gene expression of VEGF and CD1, were performed. Finally, statistical analysis of the obtained data and correlation between VEGF and CD1 genes' expression among different groups were performed.

Results: Regarding the histopathological results, the combination group, as compared to 5-FU or microvesicles treated groups, showed the most obvious improvement where numerous apoptotic bodies were observed at 24hrs, being markedly increased at 48hrs of culture, while viable cells were rarely seen. Concerning the qRT-PCR results, the combination group showed significant decrease in genes' expression of both VEGF and CD1 than the 5-FU or microvesicles treated groups. Furthermore, correlation between VEGF and CD1 genes' expression revealed a very good positive correlation.

Conclusions: it was concluded that the combined use of microvesicles and 5-FU resulted in enhanced chemosensitivity of squamous cell carcinoma cells more than the use of either 5-FU or microvesicle alone. In addition, down regulation of VEGF gene expression was associated with decreased CD1 gene expression.

KEY WORDS: squamous cell carcinoma cell line, 5-flourouracil, microvesicles, vascular endothelial growth factor, cyclin D1.

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INTRODUCTION

Globally, oral squamous cell carcinoma (OSCC) is the most common oral cancer; it arises from lip, cheek, floor of the mouth, tongue, gingiva and palate, with high mortality rate [1]. OSCC represents 90% of oral cancer, it is ordered as the sixth cancer among the common cancers worldwide [2]. OSCC has poor prognosis, because of late diagnosis and occurrence of metastasis at the time of its diagnosis [3].

5-Fluorouracil (5-FU) is a widely used anticancer drug which was utilized as chemotherapeutic agent for head and neck tumors and gastrointestinal tumors [4,5]. Its mechanism of action is related to an anti-metabolic action via suppression of thymidylate synthase (TS) and incorporating its metabolites into RNA and DNA [6] .Although 5-FU, combined to other chemotherapeutic agents, increases response rates and survival in head and neck and breast cancers; however, 5-FU has had the greatest impact in colorectal cancer[7]. Nevertheless, response rates for 5-FU as a first-line chemotherapeutic treatment for progressive colorectal cancer are only 10-15%[8]. The combination of 5-FU with more recent chemotherapies such as irinotecan and oxaliplatin has enhanced the response rates in advanced colorectal cancer to reach 40-50% [9,10].Yet; despite these developments, new therapeutic approaches are necessarily needed.

Reports about the role of mesenchymal stem cells (MSCs) in tumor development and progression have been contradictory; where **in 2009, Pinilla *et al.*[11]** demonstrated that MSCs, in co-culture in vitro experiments, stimulated the proliferation and invasion and of breast cancer cells. Besides, MSCs enhanced tumor growth and vascularization in an in vivo colorectal cancer xenograft model through increasing angiogenesis[12]. However, in addition to tumor progression, MSCs can also overwhelm tumor growth through decreasing proliferation and arresting cell cycle [13]. Anti-tumor properties are designated for MSCs derived from various sources in both in vivo and in vitro tumor models. For example, injection of MSCs in in-vivo models of hepatoma[14], prostate cancer[15] and pancreatic cancer[16] has resulted in tumor suppression. Microvesicles (MVs) derived from various cells have been established as an essential component of cell-to-cell communication involved in tissue regeneration[17], therefore they may contribute to the paracrine action of MSCs[18].

MVs are nanometer-sized, membranous vesicles secreted from many cell types into their surrounding extracellular space and into body fluids. MVs and exosomes are also found in many fluids such as: synovial fluid[19], amniotic fluid[20], bronchoalveolar lavage[21], saliva[22], plasma[23] and cerebrospinal fluid[24]. MVs, also referred to as shedding vesicles, differ from exosomes in being larger in size, about 100-1000 nm in diameter. They are formed by budding off the plasma membrane into the extracellular space[25], capturing the cellular cytosolic content in the newly formed lumen as well as plasma membrane receptors in the surrounding membrane[26].

Hence, the aim of the present work was to investigate the impact of MSCs derived MVs, either alone or in combination to 5-FU, on chemosensitivity of SCC152 cell line, through histopathological examination as well as qRT-PCR analysis to detect vascular endothelial growth factor (VEGF) and cyclin D1 genes' expression in all studied groups. Finally, statistical analysis of the obtained data and correlation between VEGF and cyclin D1 genes' expression were also performed.

MATERIAL AND METHODS

Cell Culture and Chemicals

Human squamous cell carcinoma cell line (SCC152) was purchased from the American Type Culture Collection (ATCC, CRL-3240; Minnesota, USA,). It was cultured in Dulbecco's Modified Eagle's Medium (DMEM) and enriched with 10% fetal bovine serum (FBS) and 1% concentration ratio of penicillin and streptomycin (Lonza, Verviers, Belgium). Culture of SCC152 cells were preserved

in typical humidified incubator supplied with 5% CO_2, 95% air at 37^{0}C. 5-Fluorouracil was purchased from Sigma-Aldrich Chemical Co., St. Louis, Mo, U.S.A. MVs was prepared and derived from adipose mesenchymal stem cells. 5-Fluorouracil and MVs were freshly solubilized in phosphate buffer saline (PBS). Our studied groups were included: SCC152, SCC152+5-Fluorouracil, SCC152+MVs and SCC152+5-Fluorouracil +MVs.

Microvesicles Isolation

MSCs were obtained from supernatants of human adipose mesenchymal stem cells (hAMSCs). Briefly, hAMSCs were cultured in DMEM without FBS and with added 0.5% human serum albumin (HSA) (Sigma-Aldrich) overnight. The viability of the cell cultured overnight was > 99% as detected by trypan blue exclusion. The conditioned medium was collected and stored at –80°C. The medium was centrifuged at 2,000 g for 20min to remove debris, and then ultracentrifuged at 100,000g in a SW41 swing rotor (Beckman Coulter, Fullerton, CA, USA) for one hour at 4°C. MVs were washed once with serum free M199 (Sigma-Aldrich) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH= 7.4) and submitted to a second ultracentrifugation in the same conditions. MVs were stored at –80 °C for the experiments. To quantify the protein content, the Bradford MVs were isolated from the ADSCs under non hypoxic or hypoxic condition[27].

3.2. Hematoxylin and Eosin Staining Protocol

Following fixation in 96% ethanol for 12 h, the SCC152 cells were rehydrated with graded series of decreasing ethanol concentrations, stained for 12 minutes with hematoxylin, washed with phosphate-buffered saline (PBS) for development of the blue color for 30 min, and then incubated with eosin for another 30 sec. During the next stage, the cells were washed with PBS and dehydrated using a graded series of increasing concentrations of ethanol [28].

Real time PCR

The effect of 5-FU and MVs on vascular endothelial growth factor (VEGF) and Cyclin D1 (CD1) gene's expression was assessed using real time PCR. SCC152 cells at 1x10^{5}cell/well were grown in a 6 well plate at IC50 concentration of 5-FU and MVs. Cells were washed with cold PBS, trypsinized, harvested and centrifuged. Cells were suspended in 200 µl cold RNA lysis buffer with 5 µl RNase (20 µg/ml) for 15 min. The cells were chilled on ice and further subjected to RNA extraction and purification using Thermo Fisher Scientific Inc. Germany (GeneJET, Kit, #K0732) following the manufacturer's instructions. The yield of total RNA obtained was determined at 260 and 280 nm using Beckman dual spectrophotometer. Gene's expressions were determined using real time PCR (StepOne, version 2.1, Applied biosystem, Foster city, USA). 1000ng of the total RNA from each sample were used for cDNA synthesis followed by PCR amplification cycles using SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no.PI-50217 V, UK. The thermal cycling profile was 15 minutes at 45ºC for cDNA synthesis followed by 5 minutes at 95ºC for reverse transcriptase inactivation and polymerase activation. PCR amplification 40 cycles were followed which consisted of 15 seconds DNA denaturation at 95ºC, 20 seconds primers annealing at 55 ºC and 30 second at 72˚C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of 18s RNA housekeeping gene by the ΔΔCt method. Primer sequences for each gene were demonstrated in **Table (1)**.

TABLE (1): Primers sequence of all studied genes

Gene symbol	Primer sequence from 5′- 3′
VEGF	F: GAGATGAGCT TCCTACAGCAC R: TCACCGCCTCGGCTTGTCACAT
Cyclin D 1 (CD1)	F: TGAACTACCTGGACCGCT R: GCCTCTGGCATTTTGGAG
18sRNA	F: CAGCCACCCGAGATTGAGCA R: TAGTAGCGACGGGCGGGTG

Statistical analysis:

Data were coded and entered using the Statistical Package for the Social Science (SPSS) version 22. Data were summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc tests when comparing more than 2 groups. Comparison between the two durations within each group was done using paired sample *t-test*. Correlations between quantitative variables (VEGF and CD1) were done using Pearson correlation coefficient.[29]

RESULTS

Histopathological results

Control Group:

At 24 hours, the untreated cultured SCC152 cells were highly cohesive, irregularly shaped and viable with rare apoptotic bodies **(Fig. 1a)**. Besides, following 48hrs in culture, many viable cells were observed with only few apoptotic bodies **(Fig. 1b)**.

5- FU Group:

At 24 hours, examining cultured SCC152 cells treated with 5- FU presented decreased viable cells and some apoptotic bodies **(Fig. 1c)**, while after 48hrs in culture, apparently increased apoptotic bodies with few viable cells could be noticed **(Fig. 1d)**.

MVs Group:

At 24 hours, cohesive stellate shaped viable cells were observed and few apoptotic bodies **(Fig. 1e)**. In addition, following 48hrs in culture, fewer viable cells were noticed and slightly increased apoptotic bodies **(Fig. 1f)**.

Combination Group:

At 24 hours, examining SCC152 cells after 24hrs in culture revealed numerous apoptotic bodies and only few viable cells **(Fig. 1g)**. After 48 hours in culture, abundant apoptotic bodies appeared among the cultured SCC152 while rarely viable cells could be observed **(Fig. 1h)**.

3.2. Quantitative RT-PCR and Statistical Results:

Quantitative RT-PCR analysis revealed that in the control, MVs and combination groups, VEGF gene expression wasn't significantly different in 48hrs compared to 24hrs (p-values=0.2, 0.16 and 0.56 respectively); while in the 5-FU group, significant decrease in VEGF expression occurred in 48hrs compared to 24hrs (p-value =0.013). Regarding the qRT-PCR results for cyclin D1; a statistically insignificant difference in CD1 gene expression between 48hrs and 24hrs in the control, 5-FU and combination groups (p values =0.5, 0.09 and 0.2 respectively); while in the MVs group, a significant decrease in CD1 gene expression occurred in 48hrs duration compared to 24hrs (p value= 0.02) **(Table 2)**.

Comparing the mean values ± SD of VEGF gene expression among the four studied groups showed that in 24hrs duration, no significant difference occurred between the control, 5-FU and MVs groups (p value >0.05); while a statistically significant decrease in VEGF gene expression was noticed in the combination group compared to the control and MVs groups (p-values= 0.01 and 0.02 respectively). On the other hand, in 48hrs duration, a significant decrease in VEGF gene expression occurred in the 5-FU, MVs and combination groups compared to the control one (p-values <0.001, 0.002 and <0.001 respectively) but there wasn't a significant difference between 5-FU, MVs and combination groups (p-value >0.05) **(Table 2&Fig. 2)**.

Concerning the qRT-PCR results of CD1 in 24hrs duration; a statistically significant decrease in CD1 gene expression was observed in the 5-FU and combination groups compared to the control one (p-value = 0.001), while no significant difference occurred between the MVs and control groups (p-value= 0.4). In addition, a significant increase in CD1 gene expression was observed in the MVs group compared to the 5-FU one (p-value= 0.008) but a statistically significant decrease in CD1 gene expression occurred in the combination group com-

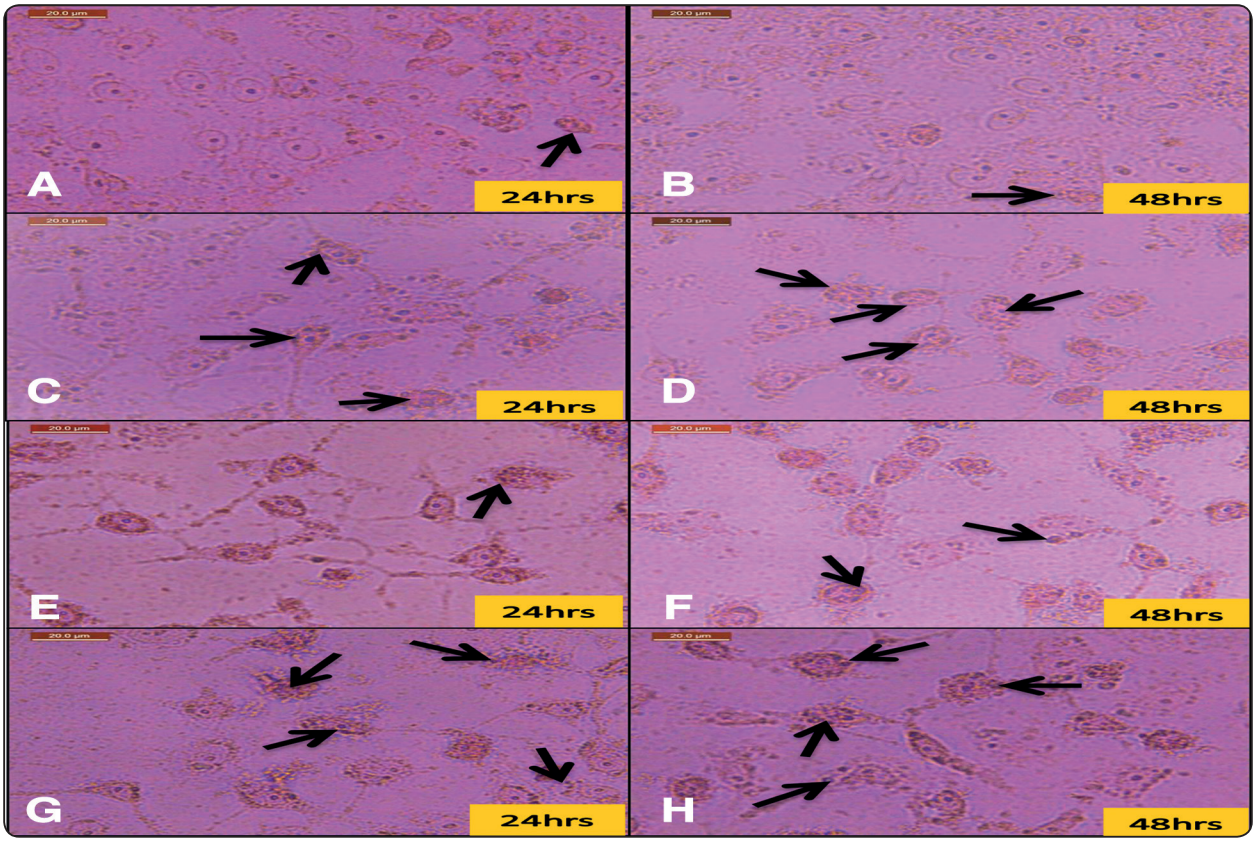

Fig. (1): Morphology of investigated SCC152 cells. Hematoxylin & eosin staining (optical magnification ×400) (a&b) Control group (a) at 24hrs of culture: highly cohesive irregularly shaped viable cells with rare apoptotic bodies (black arrow), (b) at 48hrs of culture: many viable cells with slightly increased apoptotic bodies (black arrows). (c&d) 5-FU group (c) at 24hrs of culture: decreased viable cells and some apoptotic bodies (black arrows), (d) at 48hrs of culture: abundance of apoptotic bodies (black arrows) but few viable cells. (e&f) MVs group (e) at 24hrs of culture: cohesive stellate shaped viable cells and few apoptotic bodies (black arrows), (f) at 48hrs of culture: fewer viable cells and slightly more apoptotic bodies. (g&h) Combination group (g) at 24hrs of culture: numerous apoptotic bodies (black arrows) and only few viable cells, (h) at 48hrs of culture: increased apoptotic bodies (black arrows), and rarely viable cells.

pared to the MVs one (p value 0.007). On the other hand, in 48hrs duration, there was a significant decrease in CD1 gene expression in the 5-FU, MVs and combination groups compared to the control one (p-values= 0.001, 0.007 and <0.001 respectively). However, no significant difference was noticed between the MVs, 5-FU and combination groups (p value> 0.05) **(Table 2&Fig. 3)**.

Correlation between VEGF and CD1 revealed a very good positive correlation (p-value <0.001 & r=0.879).

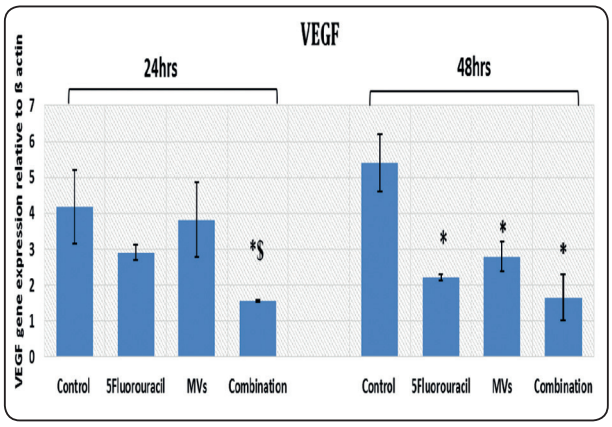

Fig. (2): A graph comparing mean ± SD of VEGF gene expression among the four studied groups. Data were expressed as Mean ± SD, p value <0.05 was significant
(*) Denotes significant difference versus control group
(#) Denotes significant difference versus 5-FLU group
($) Denotes significant difference versus MVs group

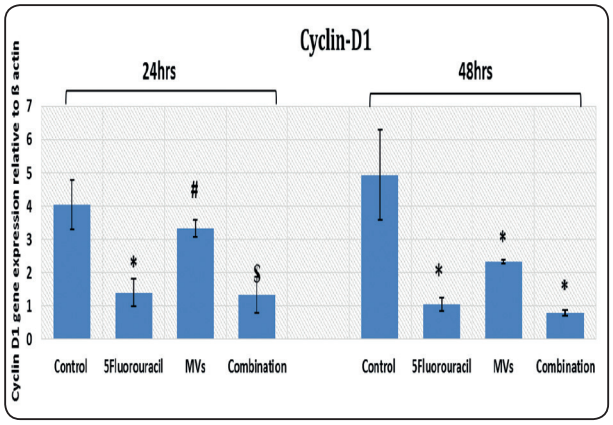

Fig. (3): A graph comparing mean ± SD of Cyclin D1 gene expression among the four studied groups. Data were expressed as Mean ± SD, p value <0.05 was significant (*) Denotes significant difference versus control group (#) Denotes significant difference versus 5-FLU group ($) Denotes significant difference versus MVs group

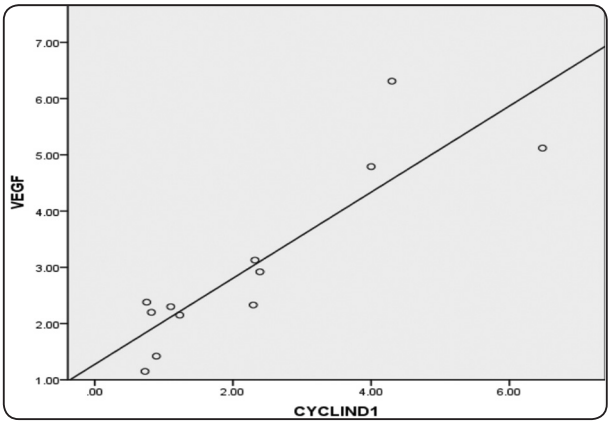

Fig. (4): A graph correlating between VEGF and Cyclin D1 genes' expressions, showing very good positive correlation between VEGF and Cyclin D1 (p-value <0.001 & r=0.879).

TABLE (2): Mean ± SD values for VEGF and cyclin D1 genes expression among the four studied groups in 24 and 48hrs durations.

In 24hrs				In 48hrs			
		Mean	SD			Mean	SD
VEGF	control	4.17	1.03	VEGF	control	5.40	.79
	5-FLU	2.90	.21		5-FLU	2.21	.07
	MVs	3.82	1.03		MVs	2.79	.41
	Combination	1.55	.032		Combination	1.65	.64
CYCLIN D1	control	4.04	.75	CYCLIN D1	control	4.92	1.35
	5-FLU	1.40	.40		5-FLU	1.05	.20
	MVs	3.33	.25		MVs	2.33	.04
	Combination	1.34	.54		Combination	.78	.08

DISCUSSION

In 2012, cancers of the lip and oral cavity represented about 2.1% of the world total, with two thirds taking place in men. The region with the utmost incidence among both males and females was by far Melanesia. Rates were likewise quite high in men in South-Central Asia (9.9) and in Central and Eastern Europe (9.1). About145, 000 deaths occurred worldwide, of which 77% occurred in the less developed regions[1]. More recently, cancer is considered the global reason of deaths more than all other diseases[30] where a new estimation made by the **WHO, in 2018,** for thirty six cancer types in 185 countries, revealed that the number of cases of lip and oral cavity cancers were (354,864) cases, 246,420 cases occurred in males, while females were less in number (108,444) and deaths were (177,384). Besides, oral cavity cancers are the most frequent type in South Asia.[31] OSCC begins by oral epithelial dysplasia, where the epithelial cells

show atypia and dysplastic changes of the tissue itself[32,33], then disruption of the basement membrane ,which is followed by invasion of tumor cells into the surrounding stroma ,causing reactive changes within the stroma[34], the biological behavior of the tumor cells is not only governed by the genetic of the tumor cells but also by the tumor microenvironment which allows progression of the tumor cells, as an outcome of the imperfect response of both epithelial and stromal components[35].

5-FU is widely used in the treatment of cancer where it causes suicidal inhibition, through irreversible hindering of thymidylate synthase inside the cells, which leads to overwhelming of DNA replication and thymineless cell loss[36].Over the past 20 years, increased understanding of the mechanism of action of 5-FU has led to the development of strategies that increase its anticancer activity. Despite these advances, drug resistance remains a significant limitation to the clinical use of 5-FU[6]. Moreover, 5-FU use has many drawbacks, as its use is accompanied by cardiotoxicity[37], fatigue, bleeding, decreased immunity, lowered blood count, diarrhea, mouth sores, hair loss, nausea, and vomiting [38,39] , in addition to the short half-life of 5-FU [40] .

Owing to the previously mentioned limitations of 5-FU use; the current study was conducted to explore the potential benefit of using MVs either alone or in combination to 5-FU in an attempt to minimize the damaging effects of prolonged use of 5-FU while increasing its anti-cancerous effect.

Based on clinical trials, mesenchymal stem cells (MSCs)-based treatment is considered rather safe, and according to our knowledge, no significant damaging effects have been described in humans. However, there are some concerns about the use of reproducing cells that may be out of control along time[41]. Preclinical studies suggested that abnormal differentiation of injected MSCs could occur. Besides, calcification of the myocardium[42] and increased accumulation of fibroblasts and myofibroblasts in the lungs [43] have been described after treatment with MSCs. As MVs hold several biological properties of the cell of origin, the development of therapeutic strategies that avoid the administration of MSCs can be imagined. This may attenuate many of the safety concerns related to the use of living cells[41]. Thus, the use of MVs could have advantages related to the use of soluble factors, the favorable effects of which are restricted by their difficult delivery to the suitable cell type[41].

Extracellular vesicles have been lately considered as key mediators of cell-to-cell communication. They can be categorized into exosomes and shedding vesicles. Exosomes originate from the endosomal membrane cell compartment then released into the extracellular space following fusion of the plasma membrane with multivesicular bodies. Shedding vesicles arise from direct budding of the plasma membrane of different cell types and are more heterogeneous in size (100nm-1 μm)[44,45]. Since both, exosomes and shedding vesicles, are found in vivo and in vitro, this diverse population is collectively called MVs[18]. MVs from various cells contain nucleic acids involving mRNA and microRNA in addition to biologically active functional proteins[46].

In the current study, histopathological examination of cultured SCC152 cells showed numerous highly cohesive irregular cells in the untreated group with rare apoptotic bodies, while following treatment with 5-FU, an obvious decrease occurred in viable cells and appearance of apoptotic bodies which increased at 48hrs of culture. In the MVs treated group, cohesive stellate shaped viable cells could be still noticed, with few apoptotic bodies which slightly increased at 48hrs of culture, while in the combination group, numerous apoptotic bodies were detected at 24hrs and were obviously increased at 48hrs of culture where viable cells were rarely seen.

The obvious improvement noticed in the combination group could be attributed to the additional anticancerous effect of MVs. The current results are in consistence with various studies

demonstrating the anti-tumor effect of MVs. For example, **in (2009) Sarkar *et al.*** demonstrated that MVs derived from lipopolysaccharide-activated monocytes could induce apoptosis in target cells through caspase-1 transfer[47]. In another study, **in (2015) Del Fattore *et al.*** [48] reported that MVs derived from cord blood and bone marrow-derived MSCs inhibited division and stimulated apoptosis in glioblastoma cells. Besides, **Yuan *et al.*** demonstrated the antitumor activity of MSCs-derived MVs loaded with recombinant tumor necrosis factor-related apoptosis-inducing ligand (rTRAIL) on their surface. In the presence of MVs, apoptosis was induced in cultured breast cancer cells. Besides, MVs did not cause apoptosis in normal human bronchial epithelial cells. Hence, the use of MSCs-derived MVs carrying rTRAIL on their surface was proven to be more efficient than using pure rTRAIL[49].

On the contrary, MVs derived from adipose MSCs induced proliferation of tumor cells [48]. Additionally, MSC-derived MVs can enhance tumor cell resistance to chemotherapeutic drugs owing to the micro-RNAs included in the MVs[50]. Thus, there are contradictory reports about the role of MSCs derived MVs in tumor development and progression and such differences might be attributed to the activation state of parental MSCs from which the MVs are derived[51].

Regarding the qRT-PCR results of VEGF gene expression, comparing all studied groups revealed that in the control, MVs and combination groups, VEGF gene expression wasn't significantly different in 48hrs compared to 24hrs; while in the 5-FU group, a significant decrease occurred in 48hrs compared to 24hrs. In addition, at 24hrs duration, no significant difference occurred between the control, 5-FU and MVs groups; while a significant decrease was noticed in the combination group compared to the control and MVs groups. On the other hand, in 48hrs duration, a significant decrease occurred in the 5-FU, MVs and combination groups compared to the control one but there wasn't a significant difference between 5-FU, MVs and combination groups.

Concerning the qRT-PCR results for cyclin D1gene expression; there was an insignificant difference between 48hrs and 24hrs in the control, 5-FU and combination groups; while in the MVs group, a significant decrease occurred in 48hrs duration compared to 24hrs. Comparing all studied groups revealed that; at 24hrs duration, a significant decrease was observed in the 5-FU and combination groups compared to the control one, while no significant difference occurred between the MVs and control groups. In addition, a significant increase was observed in the MVs group compared to the 5-FU one but a significant decrease occurred in the combination group compared to the MVs one. On the other hand, in 48hrs duration, there was a significant decrease in the 5-FU, MVs and combination groups compared to the control one. However, no significant difference was noticed between the MVs, 5-FU and combination groups. Furthermore, a very good positive correlation occurred between VEGF and CD1 genes' expression. The obtained statistical results obviously supported the current histopathological findings, where both VEGF and CD1 genes' expressions were apparently down regulated in the combination group than either 5-FU or MVs treated groups. This, in turn, back up the advantageous effect of simultaneous treatment of SCC152 cells with MVs and 5-FU compared to using 5-FU alone.

Angiogenesis is one of the most important biological processes for tumor progression, growth and metastasis[52], this process is governed by many genetic and biochemical mechanisms including: fibroblast growth factor, transforming growth factor-beta and vascular endothelial growth factor VEGF[53]. The later, being one of the most essential angiogenic factors, acts through inducing endothelial cell proliferation, survival, and vascular maturation[54]. VEGF was shown to play an important role in the angiogenesis of OSCC[55] and it has been recently considered, together with CD1, to be useful prognostic biomarkers for oral tongue squamous cell carcinoma[56]. In turn, in the herein study, VEGF and CD1 gene expressions were analyzed, statistically compared and correlated among all studied groups.

The current results revealed increased expression of VEGF gene in untreated SCC152 group while it was decreased in 5-FU, MVs and combination groups. These results are in accordance with those of **Singhal *et al.* (2016)** who demonstrated that, in cancer mucosa, VEGF is significantly overexpressed when compared to normal mucosa. The authors concluded that overexpression of VEGF was accompanied with chemo-resistance, thus; it may serve as a negative predictive marker.[57]

The well-ordered progression of cells through several phases of cell cycle (G1, S, G2, and M phases) is accurately directed by a group of proteins called "cyclins," which bind and activate cyclin-dependent kinases. Cyclin D1, a 45 kilo dalton protein encoded by cyclin D1 gene and represents a part of the molecular system regulating the cell cycle G1 to S transition[58]. Overexpression of CD1 was related to the development and progression of cancer[59].

In the present work, the expression of CD1 gene was enhanced in the SCC152 group than in the 5-FU, MVs and combination groups. These results could be supported by previous observations of **Yu *et al.*(2005)** who reported an overexpression of CD1 in head and neck squamous cell carcinoma, so, it could be a valuable prognostic marker in oropharyngeal squamous cell carcinoma[60]. Additionally, CD1 showed degradation following therapeutic anti-cancer agents use. This again could clarify the decreased expression of CD1 in 5-FU and combination groups.

Remarkably, a very good positive correlation existed between VEGF and CD1 genes' expression. This correlation could be supported by the previous findings of **Llan *et al.*(2003),** who reported that VEGF induced increased expression of CD1 and enhanced cell proliferation in hemangioma-derived cell line and primary human endothelial cells cultures, while applying an anti-VEGF neutralizing antibody in in the same cultures resulted in inhibition of both CD1 expression and cell proliferation[61]. Furthermore, in a study by **Yasui *et al.*,(2006)** they concluded that CD1 not only played a role in sustaining VEGF expression but also improved the functions of VEGF within the epithelial cells to enhance tumor vascularization[62].

Finally, according to the current results, it could be concluded that treating SCC152 with 5-FU or MVs or their combination has resulted in increased apoptotic bodies and decreased viable cells, which was most obvious in the combination group and more pronounced at 48hrs compared to 24hrs. Moreover, VEGF and CD1 genes' expression was down regulated following treatment with 5-FU or MVs or their combination, which again was markedly noticed in the combination group most. Correlating VEGF and CD1 genes' expression has revealed a very good positive correlation and thus, these two genes could be useful predictor markers for the progression and prognosis of SCC.

CONCLUSION

Despite the fact that diagnosis and treatment approaches were improved, mortality caused by oral cancers is still high. And since simultaneous use of MVs and 5-FU together in treating SCC has resulted in a remarkable reduction of viable cancer cells, increased apoptotic cell death and decreased VEGF and CD1 genes' expressions; thus, MVs represent a valuable alternative to using MSCs themselves and they can also be modified to deliver anti-tumor agents without taking the risk of using intact MSCs which; in addition to having anti-cancer properties, also have pro-oncogenic properties.

RECOMMENDATION

Preclinical animal studies and clinical trials are still needed to evaluate the long-term safety of MVs use. In addition, expression of VEGF and CD1 genes could be useful as predictive makers of tumor progression.

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