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EVALUATION OF APOPTOSIS INDUCED BY CAPECITABINE IN COMBINATION WITH GRAPHENE OXIDE NANOPARTICLES IN HAMSTER BUCCAL POUCH CARCINOMA

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ABSTRACT

Objective: current research is directed to assess apoptosis induced by capecitabine in combination with graphene oxide (GO) nanoparticles in hamster buccal pouch (HBP) carcinoma. **Subjects & methods:** 40 Syrian male hamsters 5 weeks old, weighing 80-120g, had been separated into 4 groups. **GI**: Animals left untreated. **GII:** Animals had been painted with 0.5% 7, 12-dimethylbenz (a) anthracene (DMBA) (3 times/week/14weeks). **GIII**: DMBA was applied followed by administration of capecitabine orally, 600mg/kg/day, daily for a week followed by a week of rest, then a week of treatment. **GIV**: Animals were painted with DMBA then capecitabine was administered in addition to GO, which was administered by injection directly into the tumor at a dose of 2mg/kg/day, daily for 3 weeks. At end of experiment, animals had been euthanized, then all right pouches were excised, and divided into two parts for further histologic and flowcytometric (FCM) evaluation. **Results:** Gross observation of GIV confirmed reduction in size of exophytic masses as compared to GIII. Histologic observations of GIV indicated that 5 animals exhibited well-differentiated SCC, 4 animals exhibited to GIII severe dysplasia & 1 animal exhibited moderate dysplasia compared which indicated that 6 animals exhibited well-differentiated SCC & 4 animals exhibited severe dysplasia. FCM detection of apoptosis explained that the percentage of apoptotic cells in GIV had been significantly higher than in GIII. **Conclusions:** The combination of capecitabine & GO resulted in a synergistic antitumor effect by inhibiting tumor progression and inducing apoptosis more effectively than capecitabine alone in induced HBP carcinoma.

KEYWORDS: Capecitabine, Graphene oxide, HBP carcinoma.

INTRODUCTION

Oral mucosa is the site of development oral squamous cell carcinoma (OSCC) & frequent head & neck cancer [(1)]. Around 90% of oral cancers are OSCC, which has a nearly 50% five-year mortality rate[(2)]. Chemotherapy is an important treatment scheme for oral cancer in clinical practice[(3)]. Regretfully, OSCC's prognosis remained dire. Many unwanted side effects & drug resistance are thought to be the cause.

The development of OSCC has been a multi-step process requiring initiation, promotion & progression. It may be a useful model for researching the multi-step carcinogenesis process[(4)].

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The sequence of hyperplasia, dysplasia, & carcinoma that precedes 7, 12-dimethylbenz (a) anthracene (DMBA), potent organ & site-specific carcinogen known to produce multistep carcinogenesis, is quite like the tumors that develop in patients with oral cancer [5].

One of the best-characterized tumor efficacy models, the Syrian HBP carcinogenesis model, serves as paradigm for oral oncogenesis. When topically applied 0.5% DMBA in liquid paraffin for 14 weeks, HBP carcinomas are created, & they resemble human OSCC in many ways, including morphology, histology, pre-neoplastic lesions, the propensity to invade & spread, expression of biochemical & molecular markers, & genetic & epigenetic changes [6].

Anticancer drug known as oral fluoropyrimidine nucleoside metabolic inhibitor is called capecitabine[7]. It is administered & then changed in the tumor to 5-fluorouracil (5-FU), which may stop the synthesis of deoxyribonucleic acid (DNA) & slow down the growth of the tumor [8]. It has been extensively utilized to treat several solid cancers. Additionally, it has benefits over other therapeutic drugs, including good toxicity & oral convenience [9]. The oral administration of capecitabine permits long-term daily dosing & provides the benefits of continuous 5-FU infusion without the risk of life-threatening side effects [10].

Research has concentrated on developing novel therapy approaches, such as combinatorial medication regimens for the treatment of OSCC[11]. Combined drug application may improve the therapeutic effect of chemotherapy[3]. Graphene oxide (GO) has been an oxidized version of graphene, which has been generally produced under harsh oxidation conditions [12]. GO efficiently inhibits the development of tumor spheres in several cell lines, containing those from ovarian, pancreatic, breast, lung, & glioblastoma malignancies[13]. GO nanoparticles can damage DNA and enhance generation of reactive oxygen species (ROS), which makes cancer cells more susceptible to apoptosis [14]. In this regard, main target of current research will be to evaluate apoptosis induced by capecitabine in combination with GO nanoparticles in experimentally induced HBP carcinoma.

SUBJECTS AND METHODS

Experimental animals

The procedures had been accepted by ethical & research committee protocol of Faculties of Dental Medicine, Cairo, Boys, Al-Azhar University (No. 406/1835). Forty male Syrian hamsters, aged 5 weeks and weighing between 80 & 120 grams, were acquired from Cairo University's animal house (Cairo, Egypt). Experimental animals had been kept in standard cages with sawdust bedding under controlled temperature (22±2°C), humidity (30-40%), & light (12 hours of light & 12 hours of darkness). all experimental animal was fed a regular diet and had access to water. Animal Research Unit of Faculty of Pharmacy, Cairo, Boys, Al-Azhar University is where the experiment had been carried out. The experiment was carried out according to the guidelines of medical research institutes, & international guiding principles for biomedical research containing animals [15].

Materials

DMBA 0.5% (Sigma-Aldrich company), dissolved in paraffin oil. Capecitabine had been Purchased from pharmacy as a Xeloda tablet 500mg (Genentech USA Inc. a member of Roche group). GO was Purchased as a nano-colloid (Sigma-Aldrich Company, USA).

Experimental design

After one week of adaptation animals had been separated into 4 groups of 10 hamsters in each group: GI (normal group) animals had been fed & watered only & served as negative control. GII (DMBA) in which the right HBP had been painted with 0.5% DMBA in liquid paraffin using number 4 camel's hairbrush 3 times every week, for 14 weeks, and

served as positive control [16]. GIII (capecitabine) in which DMBA was applied as in GII to induce OSCC followed by administration of capecitabine orally by plastic oral gavage, 600mg/kg/day, daily for a week followed by a week of rest, then a week of treatment [17]. GIV (capecitabine + GO) in which animals were painted with DMBA as in GII then capecitabine was administered as in GIII in addition to GO which was injected directly into the tumor by insulin syringe, 2mg/kg/ day [18].

Gross examination, sample collection, and preparation

After end of experiment, animals had been euthanized by ether inhalation, & right HBPs were excised & grossly examined. Each HBP was divided into two specimens. One specimen had been fixed in 10% neutral buffered formalin for 48 hours to be prepared for histological examination. Another part of a specimen of fresh tissue had been mechanically digested, suspended & conjugated with annexin V/ PI using an annexin staining kit as per the manufacturer's instructions.

Histological examination

Tissue samples that had been extracted, dissected & dehydrated in series of alcohol solutions. They were then cleaned in xylene, infiltrated with paraffin wax, & embedded in paraffin blocks. Sections of tissue had been cut to a thickness of 5µ & histological findings had been regularly recorded & examined using hematoxylin & eosin (H&E) staining.

FCM detection of apoptosis using annexin V/PI assay

Tissue suspension was prepared from fresh tissue specimens according to Tribukait as stated below [19]. Biopsy specimens were transported to the laboratory in isotone saline. Tumor tissue specimens were disaggregated manually, gently minced, and gently squeezed through a nylon mesh of 100µm pore diameter with isotone Tris EDTA (3.029gm of 0.1M tris hydroxymethyl aminomethane (cat. No. T-1378, sigma chemical company), 1.022gm of 0.07M sodium chloride (ADWIC) & 0.47gm of 0.005M EDTA (cat. No. E-6758, sigma) had been dissolved in 250mL of distilled water & pH had been adjusted at 7.5 using (1N HCl). Afterward, cell suspension had been centrifuged at 1800rpm for 10min, whereupon supernatant had been aspirated. After cells had been centrifuged & the supernatant was aspirated & fixed in ice-cold (ninety-six to one hundred percent) ethanol in around 1 ml for each sample.

Staining method of annexin V kit (cat. No.556547BD pharmingen FITC apoptosis Kit)

1- 100µl of cell suspensions were added onto a 5 ml test tube & then resuspended in 100ml of 1x binding buffer (1ml of 10x buffer +9ml dist. H_20). 2- 100µl of cell suspension were taken in another 5ml test tube then 5µl of annexinV (FITC label) & directly 5µl PI (Phycoerythrin label) had been added, & then incubated in dark at room temperature for 15minutes. 3- Control was prepared by incubating cells in the absence of an inducing agent. 4- The cell was ready for acquisition on FCM. The FCM analysis was performed on the Accruri C6 cytometer (Becton Dickinson (BD), Sunnyvale, CA, USA) in Mansoura Children Hospital, which has been equipped with blue & red laser, 2light scatter detectors & 4fluorescence detectors with optical filters optimized for recognition of numerous popular fluorochromes, comprising FITC (blue fluorescence) and PE (red fluorescence) with laser beam 488 nm wavelength. There were 20.000 estimated nuclei on average per specimen, & 120 nuclei were evaluated per second. Using Accuri C6 software (Becton Dickinson) for data analysis.

4quadrants (UL: upper left; UR: upper right; LL: lower left; LR: lower right) had been created for the scatterplots. Living, non-apoptotic cells in the LL quadrant are shown to be negative for both annexin V & PI. While UR quadrant displays late

apoptosis—cells positive for both annexin V & PI. UL quadrant displays dead cells, which are positive for PI but negative for annexin V. Living, early-apoptotic cells that are positive for annexin V but negative for PI are seen in LR quadrant.

Statistical analysis

Mean & standard deviation of data had been computed after statistical analysis. Version 24 of the Statistical Program for Social Science had been used to conduct one-way analysis of variance (ANOVA). ANOVA had been used in conjunction with the post hoc least significant difference test to distinguish among more than 2 distinct groups when dealing with quantitative data & parametric distribution. Utilizing appropriate probability (p-values), significance was established: The significance level is $p<0.05$, non-significant at $p>0.05$, & highly significant at $p < 0.001$.

RESULTS

Gross observations

In GI indicated no observable abnormalities. The mucosa appeared pink in color with a smooth surface during experiment period **Fig. (1A).** GII animals revealed a whitish membrane and roughened granular surface on pouch mucosa, with varying degrees of erythema & multiple exophytic nodules of variable size surrounded with an area of ulceration & bleeding. These tumor masses are usually confluent and appear as large single masses (fungating tumor masses). These large lesions had also spread to contiguous tissue so that the buccal pouch was fixed and could not be everted with the tumor. Pouch depth started to decrease & remain fixed until the end of experiment **Fig. (1B)**. GIII Animals showed marked reduction in size & number of exophytic masses as compared to GII with an area of bleeding in some animals **Fig. (1C)**. GIV animals showed a marked reduction in size & number of exophytic masses as compared to G II and GIII. There had been a significant increase in pouch length in most of the animals **Fig (1D)**.

Histopathological results

GI: Histological sections, using H&E stain showed that lining epithelium of HBP mucosa appeared normally thin with 3-5 cells thick keratinized stratified squamous epithelium. Epithelium connective tissue (CT) interface had been relatively flat with no rete processes. Basement membrane had been intact. Subepithelial CT & muscular layer had been seen **Fig. (1E)**. GII: H&E stain revealed that, 8 cases exhibited well differentiated SCC & 2 cases exhibited moderate SCC. **Fig. (1F)**. GIII: H&E stain revealed that 6 animals exhibited well-differentiated SCC & 4 animals exhibited severe dysplasia. Well-differentiated squamous cells with invading islands to underlying CT showed invasive SCC which did not invade deeply into underlying CT & nests appeared less in size with the presence of fibrosis surrounding small epithelial tumor nests, which verified regression of tumor **Fig. (1G)**. GIV: H&E stain revealed that 5 animals exhibited well-differentiated SCC, 4 animals exhibited severe dysplasia & 1 animal exhibited moderate dysplasia. Lesions frequently produced considerable amounts of keratin, had been well-differentiated, & did not invade deeply into underlying CT **Fig. (1H)**.

FCM results

Annexin V/PI assay had been performed for detection & quantification of apoptosis in all groups. It was found that apoptosis is present in GI which did not receive any treatments (mean 3.6%). After induction of cancer in GII the percentage of apoptotic cells (mean 18.3%). Treatment with capecitabine alone in GIII induced apoptosis significantly to represent (34.3%) of cells. The combination of capecitabine and GO in GIV significantly increased this percentage of GII and relation to GIII (mean 48.4%) **(Fig 2) (Table 1).** There had been highly significant difference between GIII & GIV (p-value<0.001) **(Table 2)**.

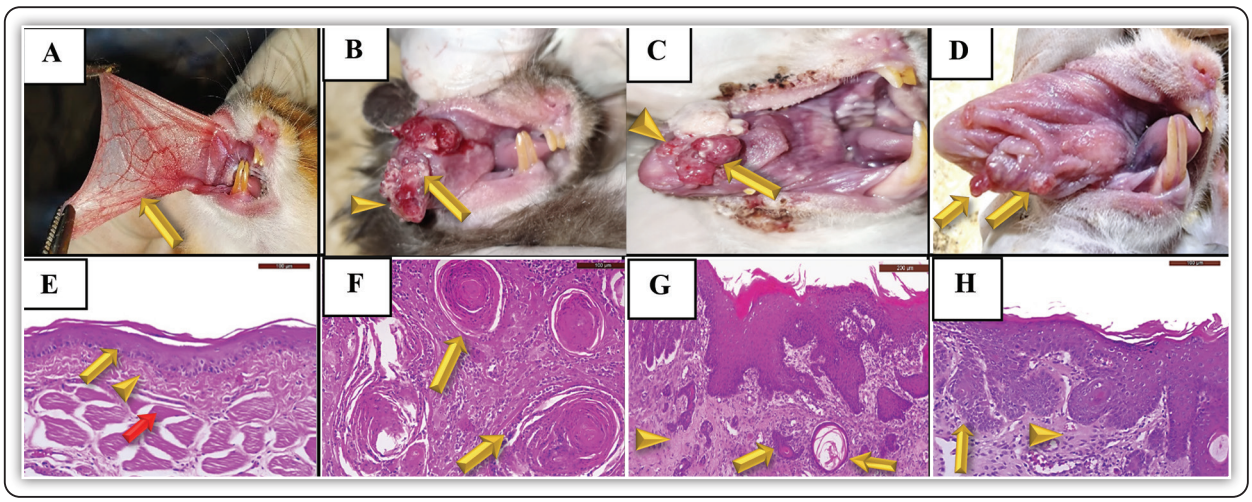

FIG (1) Gross observations & histopathological results: **Fig. (1A)**: Photograph of GI indicating normal buccal pouch with normal mucosa (arrow). **Fig. (1B)**: Photograph of GII indicating multiple large tumors (arrow) surrounded with an area of ulceration & bleeding (arrowhead). **Fig. (1C)**: Photograph of GIII indicating multiple exophytic masses with detectable tumor growth regression (arrow) and little bleeding & ulceration (arrowhead). **Fig. (1D)**: Photograph of GIV indicating multiple nodules of small size with little ulceration (arrows). **Fig. (1E)**: GI of HBP mucosa showing a defined layer of stratified squamous epithelium with flattened rete ridges (golden arrow) delicate & loose CT (arrowhead) & a layer of striated muscle fibers (red arrow) (H&E stain, x200). **Fig. (1F)**: GII of HBP mucosa indicating well-differentiated SCC with deeply invasive epithelial nests & keratin pearls (arrows) (H&E stain, x200). **Fig. (1G)**: GIII of HBP mucosa indicating well-differentiated SCC with invading islands to the underlying CT which did not invade deeply into underlying CT & nests appeared less in size (arrows). Presence of fibrous tissue surrounding nests (arrowhead) (H&E stain, x100). **Fig. (1H):** GIV of HBP mucosa showing superficial invasive SCC and revealed residual small epithelial islands (arrow). Presence of fibrous tissue surrounding nests (arrowhead). (H&E stain, x200).

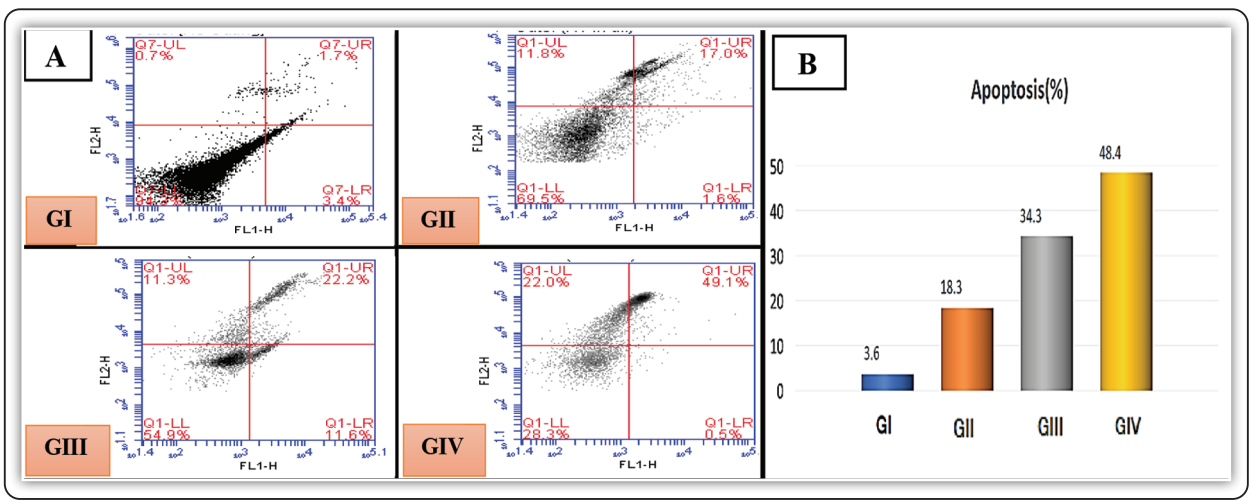

FIG (2) **(A)** Scatterplots for detection apoptosis of GI, GII, GIII, and GIV **(B)** Bar chart representing mean findings of apoptosis in studied groups.

TABLE (1) Comparisons between studied groups as regards apoptosis %.

		Pearson correlation coefficient			
I vs. II	LSD	-14.7	II vs III	LSD	-15.9
	p-value	< 0.001**HS**		p-value	< 0.001**HS**
I vs III	LSD	-30.6	II vs IV	LSD	-30.05
	p-value	< 0.001**HS**		p-value	< 0.001**HS**
I vs IV	LSD	-23.4	III vs IV	LSD	-14.08
	p-value	< 0.001**HS**		p-value	< 0.001**HS**
I vs IV	LSD	-44.7			
	p-value	< 0.001 **HS**			

DISCUSSION

With less than sixty percent of studied cases living to be older than five years, OSCC is a pathogenic kind of oral cancer that presents serious risks to health. Combination therapy is a multi-drug approach to cancer treatment. This strategy may improve the therapeutic outcomes of cancer treatment while overcoming the drawbacks of monotherapy [20]. The current study uses the HBP system as an oral carcinogenesis model to evaluate apoptosis induced by capecitabine in combination with GO nanoparticles. The evaluation had been based on gross observation, histological tumor tissue changes & annexin V FITC/ PI staining assay using FCM to evaluate apoptosis.

In current research, gross observation findings in GI (the normal group) agree with studies that reported the same findings [21,22]. This result reflected on H&E staining and agree with studies that reported the same findings [23-25]. These results due to the hamster not being exposed to the carcinogenic agent.

In this study, gross observation findings in GII (DMBA treated group) corresponding with the outcomes of studies using same model [22, 26]. These observations are because of DMBA-induced inflammation in the oral cavity. In this work, throughout the investigation, different grades of the oral tumor had been developed in animals throughout the study period. This result was corroborated by H&E staining, which revealed that DMBA-induced HBP tumors had been well to moderately differentiated SCC in form of papillomatous lesions with epithelial islands invading CT beneath. These results agree with other research works [21, 27]. In agreement with the outcomes of the present work, several studies showed that 100% tumor formation after 14 weeks of painting DMBA alone on HBP exhibits well and moderately differentiated SCC [22, 23,28,29]. This may be attributed to the procarcinogenic nature of DMBA, which induces the expression of cytochrome P 450 enzymes that metabolize DMBA into free radical intermediates, like superoxide, hydrogen peroxide, & nitrogen oxide, which could generate mutagenic DNA adduct through induction of oxidative stress and induce carcinogenesis [30].

Regarding apoptosis of all cancer cells, FCM of apoptosis results in GII revealed that 18.3% underwent apoptosis of a highly proliferative cancer tissue compared to GI 3.6%. There had been highly statistically significant variation between GI & GII regarding to apoptosis of all cancer. These results agree with another research work [31]. These results may be attributed to the fact that apoptosis was observed to gradually increase from normal to dysplasia to OSCC because of large tumor size & aggressive biological behavior. Tumor undergoes

hypoxia leading to increased apoptosis. These attributions have been consistent with those reported by Simila A et al (2018) [32].

In current research, Gross observation findings in GIII (capecitabine treated group) had been approved by H&E staining. These results were in line with another research by Arjona-Sánchez et al (2010) on experimental pancreatic cancer [33]. These results may be attributed to the fact that capecitabine is a prodrug that is selectively tumor-activated to its cytotoxic moiety. Fluorodeoxyuridine monophosphate, which is produced when 5-FU is further metabolized, binds to thymidylate synthase to form ternary complex that has been covalently bonded. This binding stops uracil from becoming thymidylate. Lack of thymidylate may avoid cell division since it has been a prerequisite for thymidine triphosphate, which has been required to produce DNA [34].

Regarding FCM of apoptosis, GIII resulted in 34.3% apoptosis. There had been a highly statistically significant variation among GIII & GII regarding apoptosis. These findings have been consistent with those reported by other investigators[35,36]. These findings may be attributed to the antitumor effect of capecitabine is to bind DNA and thereby block DNA synthesis and induce apoptosis [37, 38].

In the current research, Gross observation outcomes in GIV (Capecitabine and GO treated group) reflected on H&E staining. These findings reflected the beneficial anticancer effect of capecitabine and GO. GO paved a new avenue to use as chemosensitizer which could be potentially applied as adjuvant agents and strongly enhanced the sensitivity of chemotherapeutic agents to improve the effect of chemotherapy. The compatible finding was reported by Lin K et al (2018) [39] and Yuan Y et al (2017) [40]. Afarideh B et al (2018) showed significant inhibition of adenocarcinoma cells using GO/5-FU compared to free 5-FU [41].

Regarding FCM of apoptosis, GIV resulted in 48.4% apoptosis. There had been a highly statistically significant variation between GIV & GIII regarding apoptosis. Based on the results, the rate of apoptosis is significantly increased in cells after combination therapy of capecitabine and GO, compared to groups treated with capecitabine or GO alone. These findings did not differ much from those reported by Sanad et al (2019) [42].

CONCLUSIONS

Within the limitation of this in vivo research, the following conclusion may be drawn:

The combination of capecitabine & GO resulted in a synergistic antitumor effect by inhibiting tumor progression and inducing apoptosis more effectively than capecitabine alone in induced HBP carcinoma.

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