Al-Azhar Journal of Dental Science
Vol. 20 - No. 2 - April 2017

ELECTRON MICROSCOPIC EVALUATION OF CISPLATIN CONJUGATED NANOGOLD AND LASER IRRADIATION ON HAMSTER BUCCAL POUCH CARCINOMA

Ahmed Abdel-Shakour Abdel-Hafeze*, Mohamed Mahmoud Ahmed** *and* Mohamed Gomaa Attia Zouair***

ABSTRACT

The aim of the current study was to evaluate the ultrastructural illustration of apoptosis induced by gold nanoparticles (AuNPs) conjugated with cisplatin and laser irradiation on 7, 12- Dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouches {HBP(s)} carcinoma. **Material and methods:** The experimental animals used in the current study were fifty five golden syrian hamsters weighting 80-129 g, and obtained from the animal house, Cairo University. The experimental animals were divided into two main groups: Group A (normal group) 5 hamsters were taken, not treated and served as control. Group B: The remaining animals (50 hamsters) were treated with the same procedure: HBPs were painted for 18 weeks with DMBA, 3 times a week on alternative days, then the hamsters were randomly divided into the following five equal subgroups (10 hamsters) in each one. Group B1(DMBA treated group), group B2 (DMBA- AuNPs treated group), group B3 (DMBA- AuNPs - laser radiation treated group), Group B4 (DMBA- cisplatin-laser radiation treated group) and group B5 (DMBA- AuNPs conjugated with cisplatin - laser radiation treated group). After termination to the treatment, fresh tissues from HBP were trimmed into small pieces in order to be processed and examined by transmission electron microscopy (TEM). **Results:** compared to results of other groups, DMBA induced carcinoma in group B animals**.** AuNPs conjugated with cisplatin and laser irradiation group showed signs of cellular apoptosis ranging from shrinkage of the nuclei, degeneration of the nuclear membrane to total cell autolysis. The other remaninig groups didn`t show any signs of apoptosis. **Conclusions:** AuNPs in conjunction with cisplatin and laser irradiation illustrated various signs of cancer cell apoptosis with TEM imaging. Intra-tumoral application of AuNPs in conjunction with cisplatin and visible laser irradiation was considered as a promising therapeutic agent in regression of HBP DMBA induced carcinoma.

KEYWORDS: Squamous cell carcinoma, Apoptosis, Cisplatin, Gold nanoparticles

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises tumors of the oral cavity, pharynx and larynx, and is a relatively common human cancer. When grouped together, oral and pharyngeal cancers represent the sixth most common type of cancer worldwide[1,2]. 7, 12- Dimethylbenz (a)anthracene (DMBA) a polycyclic aromatic hydrocarbon, is widely employed to induce oral carcinoma in experimental animals including hamster buccal pouches (HBP)[3,4]. It has been found that DMBA induced oral tumors expresse biochemical and molecular characteristics similar to that of human oral tumors[5]. Among various factors, apoptosis was found to play a pivotal role in the regulation of oral squamous cell carcinoma (OSCC)[6].

The success of electron microscopy in helping to define tumor cell differentiation and promoting diagnostic precision is based on the fact that each cell has a characteristic by which it can be identified. These ultrastructures consist of distinctive and sometimes specific structures organelles and these are retained in the tumoral

* Assistant lecturer, Oral and Dental Pathology Department, Faculty of Dental Medicine (Boys-Cairo), Al-Azhar University
** Professor, Oral and Dental Pathology Department, Faculty of Dental Medicine (Boys-Cairo), Al-Azhar University, Egypt.
*** Professor and Head of Oral and Dental Pathology Department, Faculty of Dental Medicine (Boys-Cairo), Al-Azhar University, Egypt.

counterpart, even in poorly differentiated tumors. It has been found that by ultra-structural examination of OSCC revealed pathological changes in the mitochondria, lysosome, ribosome, basement membrane, cytoplasmic process, intercellular spaces and desmosomes[7-9].

Despite the significant advances made in conventional treatment for cancer such as surgery, radiotherapy, chemotherapy or combinations of them over the past decades, HNSCC continues to have a dismal prognosis, with a 5- years overall and disease-free survival of estimated ~50%[10].

Cisplatin is one of the most effective anticancer drugs and is widely used for the treatment of various malignant tumors, including HNSCC[10,11]. Apoptotic cell death of tumor cells induced by cisplatin mediated mainly via the activation of various signal transduction pathways, including calcium and death receptor signaling, in addition to activation of mitochondrial pathways[12,13].

Cancer research has been focused on improving cancer diagnosis and treatment methods via new techniques, among which are using nanoscaled particles[14]. It has been reported that there is a huge potential to use nanoparticles in cancer therapy. Nanotechnology involves the design, characterization, production and application of nanoscaled structures, devices, and systems[15]. Interestingly, updating research has proved photothermal therapy (PTT) to be a successful method for treating superficially located HBP carcinomas[16]. The most advanced area of nanomedicine is the development and use of nanoparticles for drug delivery[17-19].

MATERIAL AND METHODS

The experimental animals used in the current study were golden Syrian hamsters. They were used as model for, carcinoma induction utilizing DMBA as chemical carcinogen. Then, a direct inra-lesional injection by different kind of treatment: AuNPs, cisplatin, AuNPs conjugated with cisplatin and laser irradiation, were employed. After termination of the various treatment used, fresh tissues from hamster buccal pouche(s) ($HBP_{(s)}$) were trimmed into small pieces in order to be examined by transmission electron microscopy (TEM).

Material used

Carcinogen preparation: DMBA was prepared by dissolving 0.5gm DMBA powder in 100 ml paraffin oil (Sigma-Aldrich). **Citrate capped-AuNPs Preparation:** The brand: (NanoTech Egypt for Photo- Electronics) AuNPs was synthesized as follow: citrate stabilized spherical AuNPs with an average diameter of 20 nm were synthesized using citrate reduction method. **Cisplatin:** Platinum coordination compound from Pfizer pharmomedical company. **Cisplatin conjugated with nanogold:** Cisplatin conjugated AuNPs was, initially, prepared followed the same method as used in AuNPs preparation. **The experimental animals were classified into two main groups: Group A (normal group):** 5 hamsters were taken, not treated and served as controls. **Group B:** The remaining animals (50 hamsters) were treated with the same procedures at the beginning of the experiment. Their buccal pouches were painted for 18 weeks with DMBA, 3 times a week. Then the hamsters were randomly divided into the following five equal subgroups (10 hamsters) in each one: **Group B1(DMBA treated group)**:DMBA treated group: at 18 weeks animals were grossly examined. **Group B2 (DMBA- AuNPs treated group):**DMBA- AuNPs treated group: The lesions of this group were directly injected intra- tumoral with AuNPs (10 mg/kg) by insulin syringe. This step was repeated once every 72 hours for 9days. Then, the animals were left unhanded for 4 weeks. **Group B3 (DMBA- AuNPs - laser radiation treated group):** DMBA- AuNPs - laser radiation treated group. The lesions of this group were directly injected with AuNPs (10 mg/kg) by insulin syringe. The animals were directly exposed to light emitting diode (520-560 nm) wave length (150mw, 8mm diameter) for 15 minutes within 2 minutes

of injection to limit particle diffusion beyond the tumor boundaries. This step was repeated once every 72 hours for 9 days. Then, the animals were left unhanded for 4 weeks. **Group B4 (DMBA-cisplatin-laser radiation treated group):** DMBA-cisplatin- laser radiation treated group: The lesions of this group were directly injected with cisplatin by insulin syringe. This step was repeated in the same as previous group. **Group B5 (DMBA-AuNPs conjugated with cisplatin-laser radiation treated group)**: DMBA- AuNPs conjugated with cisplatin - laser radiation treated group: The lesions of this group were directly injected with AuNPs (10 mg/kg) conjugated with cisplatin by insulin syringe. This step was repeated same as previously.

Investigations: After 2 days of experiment, 2 animals from all groups were authanzed for tissue section preparation in order to examine the distribution of the particles of the material used in the tissue. The other 8 animals were left unhanded for 4 weeks until the end of the experiment. Fresh tissues from HBPs were trimmed into small pieces in order to be examined by TEM.

RESULTS

Group A normal group: The ultrastructural features using TEM revealed that the basal cell has normal shape with well-defined cell membrane. The mitochondria appeared within the cytoplasm in variable morphological patterns ranging from spherical to elongated shapes. The cytoplasm showed golgi apparatus and abundant fine filament in perinuclear region. The nucleus appeared oval in shape with homogenous chromatin distribution. The nucleus was seen to be surrounded with clear obvious nuclear membrane. Abundant mitochondria were detected in the perinuclear region. Numerous junctions were seen between the adjacent basal cells mostly desmosomes (Fig. 1 A).**Group B1 (DMBA treated group)**: TEM revealed that the neoplastic basal cell appeared with nucleus that has wavy nuclear membrane. A patch of condensed chromatin was seen inside the nucleus.The cytoplasm showed peripherally fine filaments, with loss of most cellular details. A destruction of intracellular junctions was seen. The neoplastic cells were seen to be attached to other by a poorly developed desmosomes (Fig. 1B). **Group B2 (DMBA-AuNPs treated group)**: TEM revealed that the neoplastic basal cell showed characteristic morphological changes with nucleus has wavy nuclear membrane. A patch of condensed chromatin was seen inside the nucleus.The cytoplasm showed peripherally fine filaments, with a loss of most cellular details. A destruction of intracellular junctions was seen as well as some cells were seen to be attached to other by a poorly developed desmosomes (Fig. 1C). **Group B3 (DMBA-AuNPs-Laser treated group)**: TEM revealed that the morphology of neoplastic basal cell appeared with prominent changes, nucleus has a wavy nuclear membrane. A patch of condensed chromatin was seen inside the nucleus. The cytoplasm showed peripherally fine filaments. A destruction of intracellular junctions was seen as well as some cells were seen to be attached to other cells by a poorly developed desmosomes (Fig. 2 A). **Group B4 (DMBA- Cisplatin-laser treated group):** TEM revealed that the neoplastic basal cell appeared with nucleus has wavy nuclear membrane. A patch of condensed chromatin was seen inside the nucleus. A destruction of intracellular junctions was seen as well as some cells were seen to be attached to other cells by a poorly developed desmosomes (Fig. 2 B). **Group B5 (DMBA-AuNPs conjugated with Cisplatin-Laser treated group):** TEM revealed that the neoplastic basal cells appeared with picknotic nucleus that has a wavy nuclear membrane. The cytoplasm showed peripherally fine filaments. The cytoplasm showed areas of hyalinization. A patch of condensed chromatin was seen inside the nucleus. A destruction of intracellular junctions was seen as well as some cells were seen to be attached to other cells by a poorly developed desmosomes. There was a loss of cellular membrane of numerous cells. Cell lysis was seen in a many huge population of cells compared to that of the other groups (Fig. 2 C).

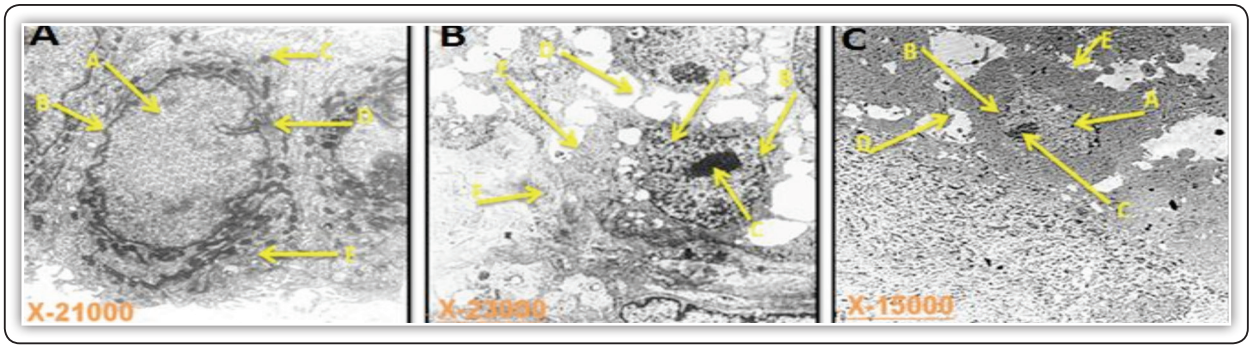

FIG.1 A: Electron micrograph of normal basal cell showing oval nucleus (arrow A), nuclear membrane (arrow B), mitochondria (arrow C) and Golgi (arrow D). Inside the cells, most of the cytoskeleton comprised fine filaments (arrow E). The mitochondria were localized in the perinuclear region. **Fig.1 B**: Electron micrograph of neoplastic basal cells in DMBA treated group showing nucleus (arrow A) with wavy nuclear membrane (arrow B), patch of condensed chromatin were observed (arrow C), poorly developed desmosomes (arrow D), interupted basal lamina (arrow E) and fine filaments (arrow F). **Fig.1 C**: Electron micrograph of neoplastic basal cells in (DMBA-AuNPs) group showing nucleus (arrow A) with wavy nuclear membrane (arrow B), patches of condensed chromatin (arrow C), poorly developed desmosomes (arrow D) and interupted a basal lamina (arrow E).

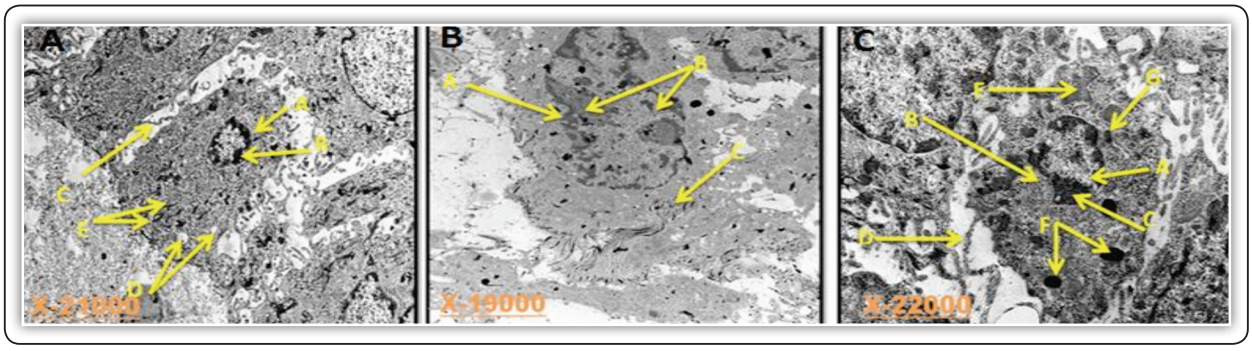

FIG.2 A: Electron micrograph of neoplastic basal cells in (DMBA-AuNPs-Laser) group showing bizarre nucleus (arrow A) filled with patches of condensed chromatin (arrows B), intra cellular fiber (arrows C) and no cell junction, desmosomes, cytoplasmic organelles and cell membrane were observed. **Fig.2B**: Electron micrograph of neoplastic basal cells in (DMBA-Cisplatin-laser) group showing pyknotic nucleus (arrow A), patches of condensed chromatin (arrow B), poorly developed desmosomes (arrow C), vacuolated mitochondria (arrows D) and fine filaments (arrows E). **Fig.2C**: Electron micrograph of neoplastic basal cells in (DMBA-AuNPs conjugated with Cisplatin-Laser) group showing pyknotic nucleus (arrow A), wavy nuclear membrane (arrow B), nucleus patches of condensed chromatin (arrow C), Cell junctions and poorly developed desmosomes (arrow D), Mitochondria (arrow E), lysosomes (arrows F) and membranous dense granular (arrow G).

DISCUSSION

The results obtained from animals treated by DMBA in the current study with those of other studies[2,3,16] support the concept that DMBA induced HBP carcinoma appeared to go through the same changes as in human. Many of the ultrastructural alterations observed in carcinogen-treated HBP mucosa closely resemble those observed during the course of human oral cancer development[20]. AuNPs were used in this work due to its ability to cross cellular membranes, when used as a drug carrier in the medical field[21]. Based on its ease of fabrication, AuNPs have a high surface area and binding capacity to target cancer cells is higher than other nanoparticles[22]. The use of AuNPs as anticancer agent was attempted by several ways as antibody conjugation, intra-tumor injection and even when painted topically alone[23]. Cisplatin and other platinum- based compounds are considered as

cytotoxic drugs which kill cancer cells by damaging DNA, inhibiting DNA synthesis and mitosis, and inducing apoptotic cell death[24].

The ultrastrctural examination of cells in group B1 revealed no signs of apoptosis. The cells showed signs of proliferation including chromatin condensation and loss of most cellular adhesions, this result run in the similarity to those of other investigators[25,26]. The complete absence of apoptosis signs in group B2 unlike group B3 was in agreement with those reported by Shamia et al (2015)[25]. The discrepancies in the results between group B2 and B3 could be due to the feasibility of laser therapy treatment on DMBA induced carcinoma. The effect of laser attributed as plasmonic AuNPs which strongly absorb light and convert it to heat energy.

In the present study, the result obtained from group B4 run in the similarity with result obtained from group B1and group B2.This provide evidence that intratumoral injection of cisplatin with laser irradiation onto HBP carcinomas did not affect the tumor cells. These results are consistent with those reported by Shamia et al (2015)[25]. In the present study, quantification of tumor apoptotic indices treated under different conditions revealed a significantly increased in B5 group compared to the groups B1, B2, B3 and B4 suggesting that photo-thermal therapy triggers cancer cell death via apoptosis. Moreover, morphological cellular changes associated with apoptosis described in the present study are consistent with those previously reported by Afifi et al (2013)[16]. Ultrastructural finding in group B5 provides evidence that the cells had all signs of apoptosis including loss of all cell details and complete cell lysis. These results are in the agreement with those observed by Afifi et al (2013)[16]. The result obtained from B5 group supports that the new strategy is to improve cancer therapy by targeting tumor cell repopulation by either the AuNPs action on tumor cells in presence of laser or using the AuNPs as vehicle for addition of cytotoxic chemotherapy. Combinatorial chemotherapy coupled with nanomedicine have opened appealing window to the current therapeutic approaches that always failed due to tumor cell resistance and unwanted side effects of drugs on normal cells, However great advances made when nano-based drug delivery systems paired with combination chemotherapeutic agents[22,25].

REFERENCES

1. Ferlay J, Isabelle S, Rajesh D, Sultan E, Colin M, Marise R, et al.: Cancer incidence and mortality worldwide: sources, methods and major patterns in globocan. Int J Cancer 2015; 136: 359–86.
2. Kempen P, Noorlag R, Braunius W, Moelans C, Rifi W.: Clinical relevance of copy number profiling in oral and oropharyngeal squamous cell carcinoma. Cancer Med. 2015; 4: 1525–35.
3. Rajasekaran D, Manoharan S, Prabhakar M, Manimaran A.: Nicostemma littorale prevents tumor formation in 7, 12 dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis. Hum Exp Tox 2015; 34: 911–21.
4. Xue T, Hua Y,Yang k, Chen D, Wang Q, Hong T, et al.: Circadian variations of clock gene Per2 and cell cycle genes in different stages of carcinogenesis in golden hamster buccal mucosa. Sci Rep 2015; 5: 97-108.
5. Jiao J, Huang L, Ye F, Shi M, Cheng X, Wang X, et al.: Cyclin D1 affects epithelial-mesenchymal transition in epithelial ovarian cancer stem cell-like cells. Onc Targets Ther 2013; 6:667-77.
6. Muzio L, Sartini D, Santarelli A, Rocchetti R, Morganti S, Pozzi V, et al.: Expression and prognostic significance of apoptotic genes in oral squamous cell carcinoma. Mol Carcinog 2012; 21: 45-52.
7. Vibha D, Hemlata T, Lakhan S: Comparison of cisplatin-based combination chemotherapy with carboplatin- based combination chemotherapy in oral and pharyngeal cancers. Int J Med Sci Public Health. 2016; 5: 497-99.
8. Yang C, Wang Y, Chun H, Yuan Z, Liu X, Yang F, et al.: IER5 promotes irradiation- and cisplatin-induced apoptosis in human hepatocellular carcinoma cells. Am Trans Res 2016; 8:1789-98.
9. Chukwuemeka V, Michael T, Steven J, Daniel J, Victoria B.: Treatment of locally recurrent and metastatic squamous cell carcinoma of head and neck. Head and Neck Cancer Res 2016; 1: 4-9.

10. Ronald P, Raghu K. Tadagavadi, Ganesan R, William B.: mechanisms of cisplatin nephrotoxicity. Toxins 2010; 2: 2491-518.

11. Florea A, Büsselberg D: Cisplatin as an anti-tumor drug: Cellular mechanisms of activity, drug resistance and induced side effects. Cancers Basel 2011; 3: 1351-71.

12. Dasari S, Tchounwou P: Cisplatin in cancer therapy molecular mechanisms of action. Eur Pharmacol 2014; 740: 364-78.

13. Kuwahara D, Tsutsumi K, Kobayashi T, Tomoko H, Nishioka k: Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. Cancer Lett 2000; 148: 65-71.

14. Jung AC, Ray A, Ramolu L, Macabre C, Simon F, Noulet F, et al.: Caveolin-1-negative head and neck squamous cell carcinoma primary tumors display increased epithelial to mesenchymal transition and prometastatic properties. Onco Target 2015; 39: 184-901.

15. Masthan K: Nanotechnology-application in oral cancer. J Clin Diag Res 2014; 7: 1328-30.

16. Afifi M, El Sheikh S, Abdelsalam M, Ramadan H, Omar T, El Tantawi M, et al.: Therapeutic efficacy of plasmonicphotothermal nanoparticles in hamster buccal pouch carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol 2013; 115: 743-51.

17. Jain S, Hirst D, Sullivan J: Gold nanoparticles as novel agents for cancer therapy. Br J Radiol 2012; 85: 101–13.

18. Xingjie W, Yanqin G, Chang M: Polymer/gold hybrid nanoparticles: from synthesis to cancer theranostic applications. RSC Adv 2015; 5: 787-96.

19. Kempen P, Greasley S, Parker K, Campbell J, Chang H, Jones J, et al.: Theranostic mesoporous silica nanoparticles biodegrade after pro-survival drug delivery and ultrasound magnetic resonance imaging of stem cells. Theranostics 2015; 5:631-42.

20. Ralf P, Marina P, Iwona C, Stefan L, Christina J, Christoph A: Novel nanoparticulate drug delivery Systems. Nanomedicine Lond 2016; 11: 573–76.

21. Amita N, Abhiney P, Rakhi G, Rajat N, Alisha S, Megha M: Comparison of immunohistochemical expression of antiapoptotic protein survivin in normal oral mucosa, oral leukoplakia, and oral squamous cell carcinoma. Pathol Res 2015; 20:793-99.

22. Patra C, Bhattacharya R, Mukhopadhyay D, Mukherjee P: Fabrication of gold nanoparticles for targeted therapy in pancreatic cancer. Adv Drug Deliv Rev. 2010; 62: 346-61.

23. Dasari S and Tchounwou P: Cisplatin in cancer therapy molecular mechanisms of action. Eur J Pharmacol 2014; 740: 364-78 .

24. Jelic S, Sotiropoulos G: ESMO guidelines working group. Hepatocellular carcinoma ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010; 21: 59-64.

25. Shamia A: Efficacy of nanogold conjugated cetuximab on hamster buccal pouch carcinoma. Master thesis, 2015; Department of Oral and Dental Paathology, Faculty of Dental Medicine, Boys, Cairo, Al-Azhar University: Cairo, Eygpt.

26. Cebrian V, Martin-Saavedra F, Yague C, Arruebo M, Santamaria J, Vilaboa N: Size-dependent transfection efficiency of pei-coated gold nanoparticles. Acta Biomaterialia 2011; 7: 3645-55.