ISSN: 0974-2115
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Chemotherapeutic effect of Ellagic acid encapsulated chitosan nanoparticles on DMBA induced hamster buccal pouch carcinogenesis

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ABSTRACT

We aimed to scrutinize the effect of EA@CS-NP on DMBA induced hamster buccal pouch tumorigenesis. To determine the inflammatory proteins, cell cycle regulatory proteins, angiogenic proteins and apoptotic proteins (intrinsic and extrinsic) by investigating some important regulators involved in the above mentioned signalling pathways. The histological microphotographs of DMBA induced hamster buccal pouch (HBP) showed squamous cell carcinoma with the keratin pearl formation with an increased Immunohistochemical expression of epidermal growth factor receptor (EGFR) and proliferative protein (Ki-67). Supplementation of EA@CS-NP to tumor bearing animals disclosed the dysplastic lesions with a decreased staining pattern of EGFR and Ki-67 expression. The protein appearance pattern such as, fas, fasL, caspase 3, caspase 8, caspase 9, PARP, Bax was found to be down regulated, perhaps the expression level of p53, Bcl2, cox-2, VEGF & cyclin D1 were markedly unregulated in DMBA induced positive control group. However, supplementation of EA@CS-NP altered the above mentioned protein expressions to near normal. Taken together, our results suggest that supplementation of EA@CS-NP inhibited the progression of HBP tumorigenesis by triggering both intrinsic and extrinsic apoptotic pathways.

KEY WORDS: Ellagic acid, Chemotherapy, Oral Cancer, Apoptosis, DMBA.

1. INTRODUCTION

Oral cancer, the most fatal neoplasms faced by the mankind today where 90 % of them are oral squamous cell carcinomas (OSCC). Jamel (2011), Conversely, at the molecular level, the genes undergo alterations, either activation of oncogenes or inactivation of tumor suppressor genes, or a combination of both results in the rapid proliferation of cancer cells, reduced cell death, tissue infiltration establishment of blood supply to the tumor, metastasis to secondary sites in the body, and finally dysfunction of affected organs De Vicente (2007). Hence, the ultimate goal of cancer therapeutics is to increase the survival time and the quality of life of the patients by reducing the unintended harmful side-effects. The most common cancer are chemotherapy, radiation and surgery with chemotherapy being the major treatments treatment modality. However, conventional chemotherapeutic agents are limited by their undesirable side effects. Therefore a compelling rationale for the development of alternative modalities for an effective drug delivery of therapeutics is highly warranted. Recently, clinical trials with many natural chemotherapeutic agents have failed due to its poor aqueous solubility and low bioavailability. One such compound Ellagic acid (EA) is naturally occurring phenolic phytonutrient ubiquitously found in numerous fruits (raspberries, strawberries, cranberries, pomegranates & grapes), nuts (wall nuts, pecans & almonds) and other plant sources. EA is a potent antioxidant, which holds well established scientific records both as a chemopreventive and chemotherapeutic agent in preclinical models against various types of cancer (Narayanan, 1999; Zhang, 1993). The versatility of EA to inhibit oral carcinogenesis through multiple pathways makes EA a potent anticancer agent. In addition, molecular targets of the EA are capable of the key regulators such as inflammatory proteins, apoptotic proteins, proliferative proteins, growth factors and angiogenesis proteins, spread across all cancer hallmarks which should make it an effective agent for prevention of cancer (Arul Mozhi, 2010), However, its poor pharmacokinetic properties hindered their clinical development. Whereas, targeted drug delivery by using nanoparticles can be used to overcome these challenges. Currently biodegradable polymeric nanoparticles are getting substantial attention for an effective drug delivery.

Among various polymeric nanoparticles, chitosan is a polymer, currently gaining considerable interest as they could control the rate of drug release, prolong the duration of the therapeutic effect and deliver the drug in targeting sites which is mainly due to its biodegradability, biocompatibility and mucoadhesive properties (Praveen and Sahoo, 2003). Furthermore the interesting feature of the biopolymer Chitosan is that it can be broken in the body by lysosomes to harmless N-acetylglucoseamine making it a highly desirable biodegradable material in forming carrier vehicles for an effective drug delivery in cancer therapy. In order to improve the chemotherapeutic efficacy of EA we synthesized Ellagic acid encapsulated Chitosan nanoparticles (EA@CS-NP) with their average particle size around 176 nm (Arulmozhi, 2013). The synthesized nanoparticles found to have A positive surface charge which can easily cooperate with the negative charge of the tumor cell membranes. Conversely, previous reports also suggest that nanoparticles around 50-200 nm exhibit passive targeting of cells through EPR (enhanced permeability and retention) effect. Danhier (2010). Hence, we aimed to investigate the mechanistic aspect of the chemotherapeutic action of synthesized EA@CS-NP on DMBA induced hamster buccal pouch tumorigenesis.

2. MATERIALS AND METHODS

Chemicals: Ellagic acid, chitosan (MW= 60-90 kDa; degree of deacetylation 85 %) and sodium tripolyphosphate (TPP), 7, 12-dimethylbenz (a) anthracene (DMBA) obtained from Sigma Aldrich, USA. Antibody's used for western blotting included ß-actin, Fas, FasL, caspases-3, caspase 8, caspase 9, PARP, Bcl-2, Bax, p53, Cox-2, cyclin D1, VEGF obtained from Santa Cruz Biotechnology, CA, USA and Neo Markers USA.

Experimental Design: Animals were randomized into 8 groups of ten animals each. Group 1 animals served as control. The left buccal pouches of the hamsters in groups 2-5 were painted with 0.5% DMBA in mineral oil using no. 4 painting brush, thrice a week from 0-10 weeks. The experimental protocol was slightly modified from Mullera et al., Mullera (2010). Each application of DMBA delivers approximately 0.5 mg of DMBA. Group 2 animals served as positive control and received no further treatment. Groups in 3-5 were treating with CS-NP (20 mg/kg b.wt), free EA (40 mg/kg b.wt) and EA@CS-NP (20 mg/kg b.wt) via oral gavages thrice a week from 11-21 weeks. Groups in 6-8 received bare CS-NP, free EA and EA@CS-NP which served as drug controls. During sacrifice by cervical decapitation after an overnight fast the left buccal pouch of each animal was harvested and the buccal tissues were excised out for histopathological, Immunohistochemical and western blot analysis.

Histopathology: The buccal tissues were fixed in 10% neutral buffered formalin for 24 hours. Then the tissues were processed and made into paraffin blocks. Thin (4-5 micron) sections were made for haematoxylin and eosin staining. Sections were incubated at 60°C for 1 hour in a hot plate and allowed to reach room temperature and then the samples were immersed in xylene for 1 to 2 minutes to dissolve the wax. The sections were hydrated through descending grades of alcohol (100 %, 90 %, 70 % and 50 %) to water and stained in Harris's haematoxylin for 3 minutes followed by washing in running tap water for 10 minutes. The sections were differentiated in 1 % acid alcohol and stained with eosin for a minute, then cleared in xylene and mounted with DPX.

Immunohistochemical staining procedure: The buccal tissue sections of 4 µm thickness were made in Poly-L-lysine pre-coated slides, care was taken to avoid any wrinkles or folding during sectioning. The sections were allowed to dry at room temperature for 24 hr. The sections were incubated at 60° C for 1 hr in hot plate, allowed to cool and then deparaffinized in xylene and brought to water through descending grades of alcohol (100 %, 90 %, 70 % and 50 %). Sections were washed in deionized water and the endogenous peroxidase activity was blocked by 3 % hydrogen peroxide for 5 min. Slides were washed twice in phosphate buffer solution (PBS) for 5 min. Slides were incubated for 5 min in 0.4 % case in PBS to block non specific reaction and then washed twice in PBS. Now the slides were placed in the staining chamber and incubated with specific anti human antibody raised in mouse for 2 hr at room temperature. Sections were washed with PBS twice for 5 min each and then incubated in 10 % (v/v) animal serum in Tris buffered saline for 30 min. Slides were washed again in PBS twice for 5 min and then incubated with nova link polymer (Anti mouse/ Rabbit IgG) for 30 min at room temperature. After washing twice in PBS, the slides were further incubated with the substrate chromogen, 3, 3' Di-amino benzidene (DAB) solution for 5 min. Finally the slides were washed in PBS twice for 5 minutes and counter stained with 0.02 % Mayer's haematoxylin and mounted with DPX. The negative control slides were stained with all the above said steps except the primary antibody. Using this technique, the expression pattern of EGFR and Ki-67 in the buccal pouch of experimental and control animals were analyzed.

3. RESULTS

Histology: Fig.1, depicts the histological photomicrograph of different stages of carcinogenesis occurred on DMBA induced tumorigenesis during the experimental period. The left buccal mucosa of the DMBA painted hamsters exhibited different stages of carcinogenesis such as hyperplasia, dysplasia, papiloma, focal carcinoma and finally well differentiated SCC. The histological microphotography of hamster buccal mucosa in control and experimental hamsters were illustrated in fig.2. In DMBA induced group, the tumor bearing hamsters exhibited well differentiated SCC with keratin pearl formation. Moreover, supplementation of EA on DMBA induced hamsters showed focal carcinoma, whereas, treatment with EA@CS-NP displayed dysplasia. Notably, CS-NP treated to tumor bearing hamsters showed moderately differentiated carcinoma. However, both control and control treated groups displayed no signs of pathological change, however, exhibiting stratified squamous epithelium with underlying smooth muscles.

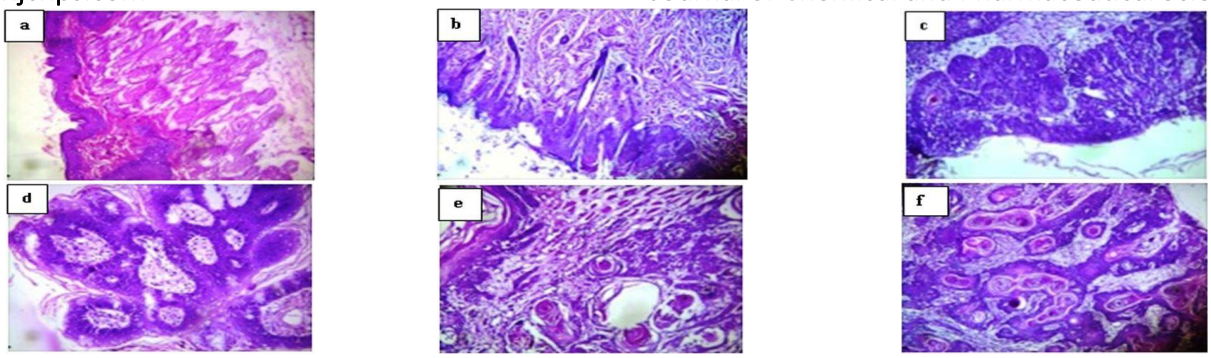

Figure.1. Histological microphotograph of different stages of carcinogenesis occurred on DMBA induced oral cancer lesions during the experimental period (0-21 weeks). a) Normal squamous epithelium, b) Hyperplasia c) Dysplasia d) Papiloma e) Focal carcinoma f) Squamous cell carcinoma

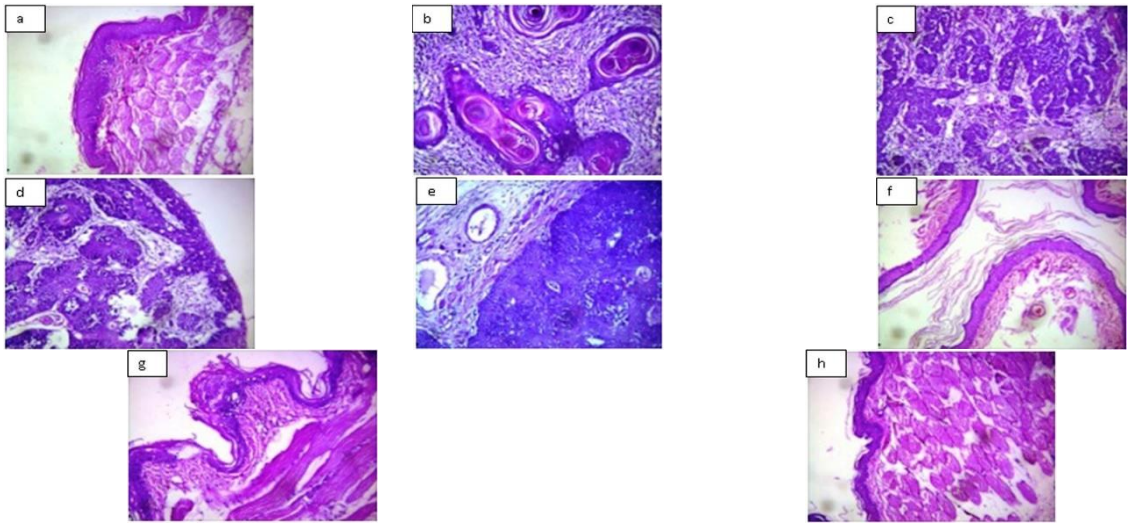

Figure.2. Histopathological microphotographs of hamster buccal mucosa in control and experimental hamsters. a) Control - Tissue lined by stratified squamous epithelium. Sub epithelial tissue show collagen and smooth muscle. No significant pathological changes, b) DMBA - Well differentiated squamous cell carcinoma with keratin pearl formation, c) DMBA+CS-NP - Moderately differentiated carcinoma, d) DMBA+EA - Focal carcinoma, e) DMBA+EA@CS-NP - Dysplasia, f) CS-NP - Normal squamous epithelium, g) EA - Strips of tissue lined by keratinized epithelium with overlying flakes of keratin, h) EA@CS-NP - Stratified squamous epithelium with underlying smooth muscle. No Dysplasia / malignancy. (Haematoxylin and Eosin staining, 10x magnification).

Immunohistochemical analysis: Fig.3. illustrates The Immunohistochemical staining of EGFR expression in the buccal pouch mucosa of control and experimental animals. Two patterns of EGFR positive staining were noticed, the majority of the positive expressions was found to have a membrane pattern of expression and few showed cytoplasmic pattern of expression. Whereas, high EGFR positive staining was observed in DMBA induced tumorigenesis compared to control group. However, treatment with EA@CS-NP exhibited less positive staining with scattered EGFR expression noted compared with free EA and CS-NP treatment. Notably, in control and control treated groups showed non-specific EGFR positivity noted in the background. Hamster buccal mucosa of control and experimental groups were stained for Ki-67 to determine the proliferation of tumor cells Fig. 4. A high rate of Ki67 positive cells was observed in DMBA induced group compared to control group. On the other, EA@CS-NP showed week Ki67 immunoreactivity in comparison with free EA and CS-NP. Of note, both control and control treated hamsters possessed negative Ki67 staining.

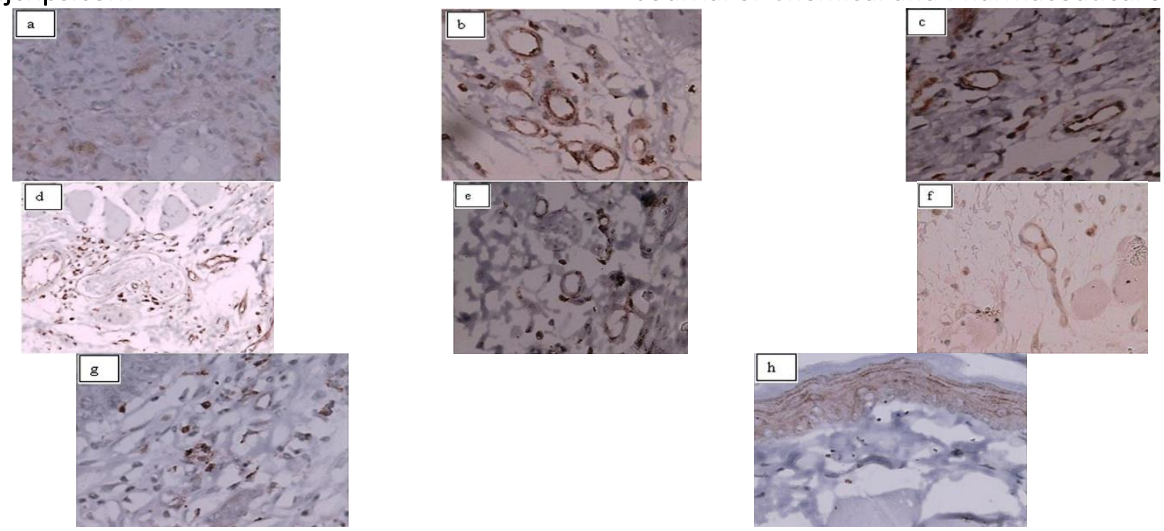

Figure.3. Immunohistochemical staining pattern of EGFR in buccal mucosa of control and experimental animals. a) Control - Non-specific positivity noted in the background, b) DMBA - Positive staining (high EGFR positivity observed in the tumor or lesion cells). c) DMBA + CS-NP - Positive staining of EGFR noted in the tumor cells, d) DMBA + EA - EGFR positive staining was observed, e) DMBA +EA@CS-NP - Scattered positivity noted, f) CS-NP – Non-specific positivity noted, g) EA - Negative staining (non-specific background staining), h) EA@CS-NP - Non-specific positivity noted in the background

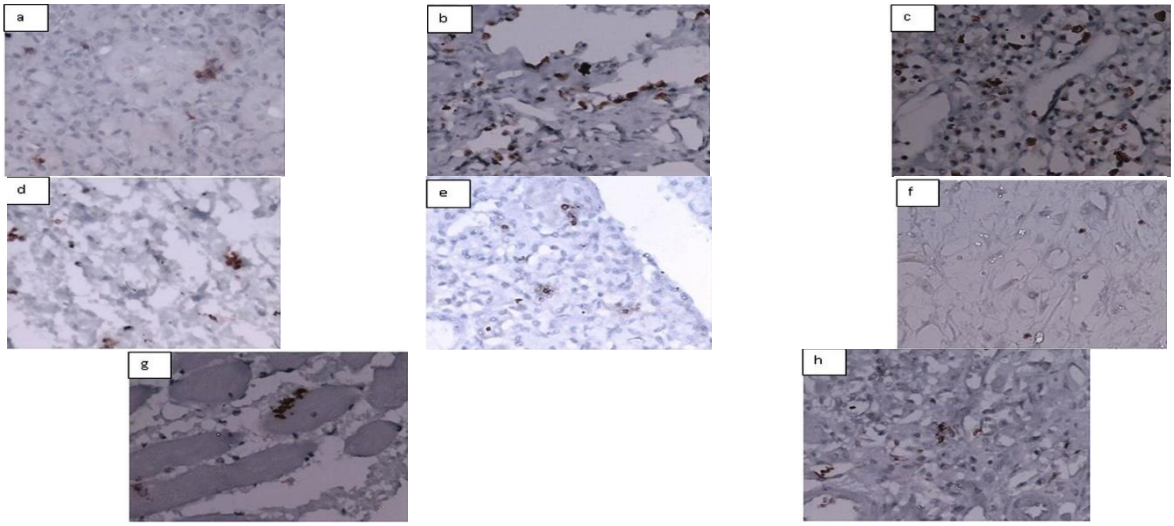

Figure.4. Immunohistochemical detection of Ki67 expression in buccal mucosa of control and experimental animals. a) Control - Negative non-staining, b) DMBA - High positive staining (27 %), c) DMBA + CS-NP - Positive Ki-67 (14 %), d) DMBA + EA - Positive Ki-67 (6.5 %), e) DMBA + EA@CS-NP - Low Positive Ki-67 (0.2 %), f) CS-NP Negative Ki-67, g) EA - Negative Ki-67, h) EA@CS-NP -Non-specific negative staining.

Western blot analysis: Western blot analysis illustrates the molecular changes occurred during tumorigenesis. We sought to determine the inflammatory proteins, angiogenic proteins, cell cycle regulatory proteins and apoptotic proteins, by investigating some important regulators involved in the above signalling pathways fig. 5-9. The expression pattern of the proteins such as fas, fasL, caspase 3, caspase 8, caspase 9, PARP, Bax was found to be down regulated whereas p53, Bcl-2, cox-2, VEGF and cyclin D1 were markedly up regulated in DMBA induced positive control group. In CS-NP treatment of tumor bearing animals, the expression of proteins were more or less the same as that of group 2 hamsters. However, it is noteworthy that supplementation of EA and EA@CS-NP to tumor bearing animals remarkably altered the expression of markers to near normal when compared to group 2 positive control. It is of note that compared to EA, EA@CS-NP exhibited increased protein expressions, and no significant changes were observed in control and control treated groups.

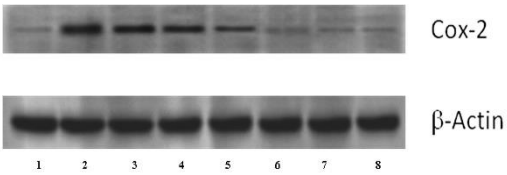

Figure.5. Representative blot analysis of inflammatory protein expression Cox-2 in HBP tissue. Group 1 – control, 2 – DMBA, 3 – DMBA + CS-NP, 4 – DMBA + EA, 5 – DMBA + EA@CS-NP, 6. CS-NP, 7 – EA, 8 – EA@CS-NP

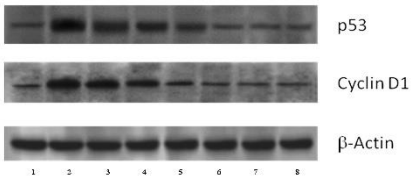

Figure.6. Representative blot analysis of cell cycle regulatory protein expression p53 and Cox-2 in HBP tissue. Group 1 – control, 2 – DMBA, 3 – DMBA + CS-NP, 4 – DMBA + EA, 5 – DMBA + EA@CS-NP, 6. CS-NP, 7 – EA, 8 – EA@CS-NP

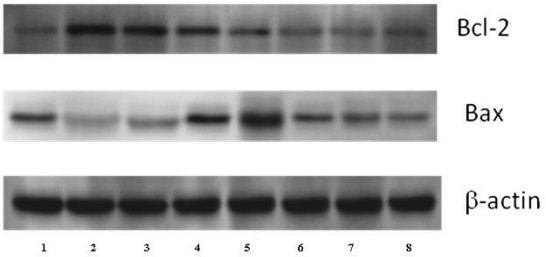

Figure.7. Representative blot analysis of proapoptotic and antiapoptotic protein expression Bax and Bcl-2 in HBP tissue. Group 1 – control, 2 – DMBA, 3 – DMBA + CS-NP, 4 – DMBA + EA, 5 – DMBA + EA@CS-NP, 6. CS-NP, 7 – EA, 8 – EA@CS-NP

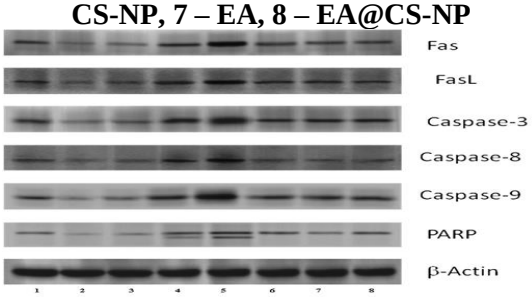

Figure.8. Representative blot analysis of apoptotic protein expression Fas, FasL, caspase-3, Caspase-8, Caspase-9, PARP in HBP tissue. Group 1 – control, 2 – DMBA, 3 – DMBA + CS-NP, 4 – DMBA + EA, 5 – DMBA + EA@CS-NP, 6. CS-NP, 7 – EA, 8 – EA@CS-NP

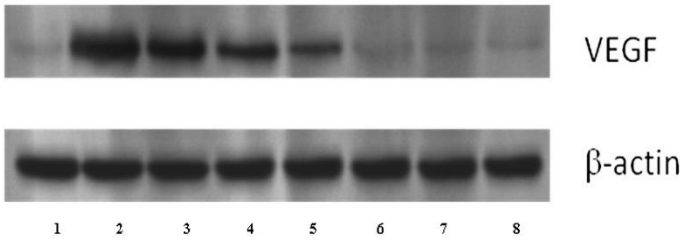

Figure.9. Representative blot analysis of angiogenic protein expression VEGF in HBP tissue. Group 1 – control, 2 – DMBA, 3 – DMBA + CS-NP, 4 – DMBA + EA, 5 – DMBA + EA@CS-NP, 6. CS-NP, 7 – EA, 8 – EA@CS-NP.

DISCUSSIONS

Chemotherapeutic mechanism of EA@CS-NP was investigated in DMBA induced hamster buccal pouch carcinogenesis. The pathological status of tumorigenesis was identified by altered the cell morphology and tissue architecture in HBP. During the experimental period, an array of pathological changes was observed in DMBA induced tumorigenesis, which exhibited hyperplasia, dysplasia, papiloma, focal carcinoma and OSCC. DMBA induced group exhibited well differentiated SCC with keratin pearl formation. Whereas, supplementation of EA@CS-NP to tumor bearing hamsters significantly delayed the tumors, which showed dysplastic lesions, however, bare CS-NP, hence our result validates the pronounced chemotherapeutic potential of EA@CS-NP. In our laboratory, we scrutinized the 3, 3'-Diindolylmethane encapsulated Chitosan nanoparticles [DIM@CS-NP] on DMBA induced Sprague Dawley rats by evaluating oxidant, antioxidant and histophathological changes (Isabella, 2016).

Epidermal growth factor receptor (EGFR), is an imperative biomarker and useful prognostic indicator of oral cancer. It plays, a key role in cell signal transduction and tumor growth, which contributes to proliferation, invasion and metastasis formation. A high frequency of EGFR over expression reported in cancers of the head and neck region (Shane, 2016). Conversely, our IHC demonstration of EGFR over expression in DMBA induced HBP tumorigenesis was in agreement with the earlier reports whose EGFR expression was found to be markedly enhanced in OSCC (Shang, 2008). The possible reason could be that increased expression of EGFR may be related to the degree of differentiation of neoplastic keratinocytes which led to the aggressive tumor formation. These increased expressions tend to have more rapid cell cycle progression, greater chemo resistance, inhibition of apoptosis, increased angiogenesis, cell mortality and elevated metastasis (Nagini, 2009; Inoue, 2012). Notably, treatment with CS-NP to tumor bearing hamsters showed increased EGFR expression and no significant variations in the staining was observed when compared to DMBA induced group which indicates poor prognosis. In addition, quite increased

EGFR positive staining was noticed in free EA treated tumor bearing animals. Interestingly, supplementation of EA@CS-NP to DMBA induced tumorigenesis exhibited less scattering of EGFR protein expression compared to other DMBA induced tumor bearing groups implicating better prognosis. Moreover, previous evidence indicated EA as a tyrosine kinase EGFR inhibitor in cervical and lung cancer cells (Rajkishen, 2006). Hence it is apparent from our result that nanoformulated EA inhibits EGFR expression to a greater extent when compared to free EA which results in increased prognosis of the disease. Tumor cell proliferation activity is believed to reflect a tumors biological aggressiveness, in oral cancer, prognostic significance depends on the cell proliferation rate (Tubiana, 1989).

Hence, Ki-67 an important proliferative marker that can be used to assess the percentage of cycling cells in tumor tissues. The immunostaining of Ki-67 provides a reasonable estimate of the degree of cellular proliferation. In the present investigation, increased nuclear staining of Ki-67 was observed in DMBA induced well differentiated tumors of the positive control group. It is noted that treatment with CS-NP and free EA on tumor bearing animals also showed increased nuclear staining of Ki-67 expression proving that there was no satisfactory prognosis of the disease. Treatment with EA@CS-NP on tumor bearing animals showed decreased Ki-67 expression compared to other DMBA induced experimental and control groups. The recent clinical data which highlights that patients bearing tumors with high Ki-67 were associated with high rates of disease recurrence and short survival time, whereas those having low Ki-67 were associated with a low rate of disease recurrence and long survival time (Eramah, 2012). From the above report it is clear that EA@CS-NP treatment showing a low rate Ki-67 expression can result in improved prognosis of the disease with long survival.

Cyclooxygenase (COX) is the rate limiting enzymes that catalyze the conversion of arachidonic acid to prostaglandins (PGs). In humans and experimental animals prostaglandins are believed to play a key role in the development and progression of many cancers (Pandey, 2008). Based on the previous report in both clinical and experimental studies, we found that, COX-2 may be a crucial factor in the development of OSCC (Husvik, 2009; Mccormick, 2010) and is usually associated with poor prognosis and short survival. In our study, we observed an increased expression of COX-2 in HBP of DMBA induced positive control group, the increased COX-2 expression contribute to malignant transformation and tumor growth. Segawa (2008). In their study suggested that, the expression of Cox-2 was also found to be increased in CS-NP treated tumor bearing animals and there was no significant changes between DMBA induced positive control group. Notably, free EA treated group showed slight decrease of Cox-2 expression, however EA@CS-NP supplemented group markedly decreased the expression of Cox-2 compared to DMBA induced group which indicates suppression of tumor development.

p53 gene stands out as a key tumor suppressor and a master regulator of various signaling pathways which plays, a vital role in inducing apoptosis and acts as cell cycle checkpoints in humans and murine cells following DNA damage (May and May, 1999). The mutation of p53 gene contributes to tumor initiation, promotion, aggressiveness and metastasis. In most cancers, mutation of the tumor suppressor gene p53, significantly contributes to cancer development. Further, over expression of p53 in the buccal mucosa of tumor bearing animals suggests an accumulation of mutant p53 protein during DMBA induced oral carcinogenesis (Gimenz-conti, 1996). In connection with the above findings, we found an increased p53 expression in DMBA induced group which indicates the uncontrolled cell proliferation and a loss of inducing apoptosis. (Lane, 2011; Kastan, 1995). CS-NP treated tumor bearing hamsters did not show any significant change in p53 expression when compared to DMBA induced group. However, treatment with free EA to tumor bearing animals showed a slight dwindle the expression of p53. Noteworthy, a remarkable decrease of p53 expression was observed in EA@CS-NP treated tumor bearing hamsters which are evidenced to induce apoptosis and proves to act as cell cycle checkpoints. Cyclin D1 an oncogene encoding with a positive regulator of G1 phase progression through the cell cycle that regulates the initiation of DNA synthesis. It binds to and activates its kinase partners CDK4 and CDK6 resulting in the phosporylation of the retinoblastoma protein, thereby induces transcription of genes that promote progression to the S-phase of the cell cycle (Baker, 2005). In addition, there is a growing evidence that dysregulation of cyclin D1 plays an important role in oral SCC development which is frequently amplified and over expressed (Even and Lamb, 2004). In 25-70 % of oral cancers and a high percentage of premalignant lesions, suggesting that cyclin D1 gene amplification and consequent protein over expression which found early event during tumorigenesis (Akerall, 1997). Furthermore, cyclin D1 over expression has been associated with more aggressive tumor behavior and a worse prognosis. In the current investigation, over expression of cyclin D1 was observed in HBP tumorigenesis is consistent with the findings of DMBA induced experimental tumors and human OSCC patients (Vidya Priyadaesini, 2012). However, diminished expression of cyclin D1 was found in EA@CS-NP treated tumor bearing hamsters compared to other DMBA induced groups resulting in inhibition of proliferating cells. Our findings supports well with a recent report, stated that dietary intake of EA down control the expression of cyclin D1 in DMBA hamster buccal pouch model (Vidjaya Lechomy, 2006). Hence, our study suggests that, treatment with nanoencapsulated EA effectively enhance their prognostic role by decreasing the cyclin D1 expression.

Vascular endothelial growth factor (VEGF), is a powerful angiogenic cytokine involved in every stage of vascular development. It increases vessel permeability and enhances endothelial cell growth, proliferation, migration and differentiation (Shane, 2006; Ferrara, 2003). VEGF positive oral carcinomas is significantly poorer and patients with VEGF positive tumors are three times more likely to die when compared to patients with VEGF negative. It is not work that the observed increase in DMBA induced VEGF protein in HBP tumorigenesis is in agreement with other reports in hamsters and the oral cancer patients (Siddavaram, 2012; Shang, 2002). However, EA@CS-NP supplemented group remarkably reduced the VEGF expression to near normal when compared to other DMBA induced groups. It is evident from the previous literature that, inhibition of VEGF results in an increased mortality, impaired organ development and impaired bone cartilage vascularisation. Ferrara (2003). Hence, we revealed that nanoencapsulated EA inhibits the development of angiogenesis resulting in suppression of tumors which could decrease mortality.

Moreover, apoptosis could generally be mediated by two major cellular signalling pathways: intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. In the intrinsic pathway, mitochondria are the key mediators of apoptosis. Activation of the intrinsic pathway is mainly regulated by the Bcl-2 family proteins which consist of both pro and antiapoptotic molecules to maintain the balance of cell death and survival in a cell (Scovassi and Poirier, 1999). The targeting apoptotic pathways using nanoencapsulated EA can be an outstandingly hold back the promotion and progression stages on DMBA induced HBP carcinogenesis. Moreover, apoptosis could generally be mediated by two major cellular signalling pathways: intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. In the intrinsic pathway, mitochondria are the key mediators of apoptosis. Activation of the intrinsic pathway is mainly regulated by the Bcl-2 family proteins which consist of both pro and antiapoptotic molecules to maintain the balance of cell death and survival in a cell (Scovassi and Poirier, 1999). Antiapoptotic Bcl-2 family members (Bcl-2) can block mitochondrial events, whereas proapoptotic Bcl-2 family members (Bax) can eventually trigger those changes. This is an interesting feature where, down regulation of Bcl-2 protein has been proposed as a new promising treatment strategy for oral cancer. Hence, the above mentioned phenomenon helps in releasing cytochrome c from mitochondria into the cytosol, which is recruited into the death squad and contribute to apoptotic dismantling of the cell.

Further, cytochrome c binding with Apaf1 leads to the activation of active Caspase 9 by forming an apoptosome complex, which further triggers the activation of Caspase 3 which is considered as a key executor for apoptosis. The Bcl-2 family member Bid provides the link between the caspase signaling cascade and the mitochondria. Caspase-8 cleaves Bid in its truncated form (tBID) which in turn translocates to the mitochondria where it acts in combination with the pro-apoptotic Bcl-2 family member proteins to induce the release of cytochrome c and other mitochondrial proapoptotic factors like Smac/Diablo into the cytosol. We have focused on the apoptotic proteins involved in both intrinsic and extrinsic signalling pathways. Our results reveal the augmentation of proapoptotic protein Bcl-2 which is followed by a diminished expression of the antiapoptotic protein bax in DMBA induced group which resulted in increased bax and bcl-2 ratio. Consequently, both intrinsic and extrinsic proteins such as fas, fasL, caspases 3, caspase 8, Caspase 9, PARP and Bax showed decreased expression in DMBA induced HBP tumorigenesis, which was in agreement with earlier reports. Nagini (2009). Notably treatment with CS-NP has not shown any significant changes in the apoptotic protein expressions compared to DMBA induced positive control group. However, supplementation of both EA and EA@CS-NP to tumor bearing animals significantly up regulated the expression of Bax and down regulated the expression of Bcl-2 thereby decreases the Bax/Bcl-2 ratio. In addition, they effectively altered the apoptotic proteins to near normal levels, thereby suggesting the involvement of both mitochondrial dependent and death receptor mediated apoptotic signaling pathway. Noteworthy, increased apoptotic protein expressions were observed in EA@CS-NP treated tumor bearing group compared to free EA treated group. This may be that CS acted as a promising drug carrier which enhanced the anticancer property of the polyphenolic compound Ellagic acid.

4. CONCLUSION

Hence, EA@CS-NP have superior therapeutic activity in tumor bearing buccal tissues by actively supplying EA molecules for prolonged periods through a sustained release manner, thereby exerting an enhanced antitumor effect on DMBA induced hamster buccal pouch tumorigenesis. Our results provide evidence that EA@CS-NP induced apoptosis via both intrinsic and extrinsic pathways which will serve as an effective chemotherapeutic agent for the treatment of oral cancer in humans.

5. ACKNOWLEDGEMENT

This study was supported by the University Grants Commission, New Delhi, India, in the form of Research Fellowship in Sciences to Meritorious Students (RFSMS), No. H2/SCH/2010.

Conflict of Interest: The authors declare that there are no conflicts of interest.

REFERENCES

Akervall J.A, Michalides R.J, Mineta H, Amplification of cyclin D1 in squamous cell carcinoma of the head and neck and the prognostic value of chromosomal abnormalities and cyclin D1 overexpression, Cancer, 79, 1997, 380–389.

Arulmozhi V, Mirunalini S, Ellagic acid, A novel polyphenolic antioxidant and their therapeutic applications. Pharmacologyonline, 3, 2010, 446-457.

Arulmozhi V, Pandian K, Mirunalini S. Ellagic acid encapsulated chitosan nanoparticles for drug delivery system in human oral cancer cell line (KB). Colloids Surf B, Bioint, 110, 2013, 313-320.

Baker GL, Landis M.W, Hinds P.W, Multiple functions of D-type cyclins can antagonize pRb-mediated suppression of proliferation, Cell Cycle, 4, 2005, 330– 338.

Danhier F, Feron O, Preat V, To exploit the tumor microenvironment, Passive and active tumor targeting of nanocarriers for anticancer drug delivery, J Controlled Release, 148, 2010, 135-146.

De Vicente J.C, Lequerica-Fernandez P, Santamaria J, Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis, J Oral Pathol Med, 36, 2007, 415–424.

Eramah E, Abdelbaset B, Fathi A, Prognostic value of proliferation markers, Immunohistochemical Ki-67 expression and cytometric S-phase fraction of women with breast cancer in Libya. J Cancer, 3, 2012, 421-431.

Even M.E, Lamb J, The activities of cyclin D1 that drive tumorigenesis, Trends Mol Med, 10, 2004, 158-162.

Ferrara N, Gerber HP, LeCouter J, The biology of VEGF and its receptors, Nat Med, 9, 2003, 669–676.

Gimenz-conti IB, La Bate M, Liu F, p53 alterations in chemically induced hamster cheek-pouch lesions, Mol Carcinog, 16, 1996, 197-202.

Husvik C, Khuu C, Bryne M, PGE_2 production in oral cancer cell lines is COX-2 dependent, J Dent Res, 88, 2009, 164-169.

Inoue S, Patil R, Portilla Arias, Nanobiopolymer for Direct Targeting and Inhibition of EGFR Expression in Triple Negative Breast Cancer, Plos One, 7, 2012, 31070.

Isabella S,Mirunalini. S, Chemotherapeutic effect of 3,3′-Diindolylmethane encapsulated chitosan nanoparticles on7,12-Dimethylbenz (a) anthracene induced mammary cancer – A dose dependent study, New Horizonsin Translational Medicine, 3, 2016, 1–8.

Jamel A, Bray F, Centre MM, Global cancer statistics, CA-Cancer, J Clin, 61, 2011, 69-90.

Kastan M.B, Canman C.E, Leonard C.J, p53, cell cycle control and apoptosis, implications for cancer, Cancer Metastasis Rev, 14, 1995, 13-15.

Lane D.P, The regulation of p53 function, Steiner Award Lecture, Int J Cancer, 57, 1994, 623-627.

Maeda T, Matsumura S, Hiranuma H, Jikko A, Furukawa S, Ishida T, Expression of vascular endothelial growth factor in human oral squamous cell carcinoma, its association with tumor progression and p53 gene status, J Clin Pathol, 51, 1998, 771–775.

May P, May E, Twenty years of p53 research, structural and functional aspects of the p53 protein, Oncogene, 18, 1999, 7621–7636.

McCormick D.L, Phillips J.M, Horn T.L, Overexpression of cyclooxygenase-2 in rat oral cancers and prevention of oral carcinogenesis in rats by selective and nonselective COX inhibitors, Cancer Prev Res, 3, 2010, 73-81.

Mullera K, Zucolotob S, Juniorc R.F.A, Lack of inhibitory effect of lycopene on dysplastic lesions induced by DMBA in hamster buccal pouch, Nut Res, 27, 2007, 574–579.

Nagini S, Vidjaya Letchoumy P, Thangavelu A,Humans and hamsters, A comparative evaluation of carcinogen activation, DNA damage, cell proliferation, apoptosis, invasion and angiogenesis in oral cancer patients and hamster buccal pouch carcinomas, Oral Oncol, 45, 2009, 31-37.

Narayanan B.A, Geoffroy O, Willingham, p53/ p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells, Cancer Lett, 136, 1999, 215-221.

Pandey M, Prakash O, Santhi W.S, Overexpression of COX-2 gene in oral cancer is independent of stage of disease and degree of differentiation, Int J Oral Maxillofac Surg, 37, 2008, 379-383.

Parveen S, Sahoo SK, Long circulationg chitosan/PEG blened PLGA nanoparticle for tumor drug delivery, Eur J Pharmacol, 670, 2011, 372-383.

Rajkishen N, Christina H, Dominick N, Fifth AACR International Conference on Frontiers in Cancer Prevention Research, 11, 2006, 12-15.

Sahoo S.K, Labhasetwar V, Nanotech approaches to drug delivery and imaging, Drug Discov Today, 8, 2003, 1112–1120.

Scovassi AI, Poirier GG. Poly (ADP-ribosylation) and apoptosis, Mol Cell Biochem, 199, 1999, 125–127.

Segawa E, Sakurai K, Kishimoto H, Expression of cyclooxygenase-2 and DNA topoisomerase II alpha in precancerous and cancerous lesions of the oral mucosa, Oral Oncol, 44, 2008, 664-671.

Shane J, Richard M.L, The role of vascular endothelial factor (VEGF) in oral dysplasia and oral squamous cell carcinoma, Oral Oncol, 42, 2006, 337-342.

Shang J, Shui Y, Sheng L, Epidermal growth factor receptor and human epidermal growth receptor 2 expression in parotid mucoepidermoid carcinoma, possible implications for targeted therapy, Oncol Rep, 19, 2008, 435-440.

Shang Z.J, Li J.R, Li Z.B, Circulating levels of vascular endothelial growth factor in patients with oral squamous cell carcinoma, Int J Oral Maxillofac Surg, 31, 2002, 495-508.

Siddavaram N, Ramamurthi V.P, Veeran V, Chlorophyllin abrogates canonical Wnt/-catenin signalling and angiogenesis to inhibit the development of DMBA induced hamster cheek pouch carcinomas, Cell Oncol, 35, 2012, 385-395.

Tubiana M, Courdi A, Cell-proliferation kinetics in human solid tumors, relation to probability of metastatic dissemination and long-term survival, Radiother Oncol, 15, 1989, 1- 18.

Vidjaya Letchoumy P, Mohan V.P.C, Prathiba D, Comparitive evaluation of antiproliferative, antiangiogenic and apoptosis inducing potential of black tea polyphenols in the hamster buccal pouch carcinogenesis model, J Carcinog, 6, 2007, 1-9.

Vidya Priyadarsini R, Kumar N, Nagini S, Gene expression signature of DMBA induced hamster buccal pouch carcinomas, Modulation by chlorophyllin and Ellagic acid, Plos One, 7, 2012,1-9.

Zhang Z, Hamilton S.M, Stewart C, Inhibition of liver microsomal cytochrome P450 activity and metabolism of the tobacco-specific nitrosamine NNK by capsaicin and ellagic acid, Anticancer Res, 13, 1993, 2341-2346.