Effects of aqueous cinnamon extract on chemically-induced carcinoma of hamster cheek pouch mucosa

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\textbf{ABSTRACT}

This study aimed to investigate the effects of aqueous cinnamon extract (ACE) on 7, 12-Dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch (HCP) mucosa. Sixty male Syrian hamsters were randomly divided into six equal groups. The hamsters of groups I, II and III received no treatment, DMBA and ACE respectively, for 16 weeks. Groups IV and V were handled as group II and concomitantly treated with ACE for the same period and additionally group V received ACE for other 16 weeks after the stoppage of DMBA application. Group VI hamsters were handled as group III and additionally received DMBA for other 16 weeks after the stoppage of ACE supplementation. Hamsters of each group were euthanized according to the experimental schedule. The buccal pouches were and prepared for H & E stain, PAS reagent, CD3 and PDGF immunohistochemical reactivity. All groups showed dysplastic changes with varying degrees except groups I and III. Deep invasive carcinomas were recorded in 90% of the samples of group II, 60% of group IV, 50% of group V and 40% of group VI. From the previous results, it can be concluded that ACE has the potentiality preventing oral cancer initiation better than inhibiting oral cancer progression.

\section{1. Introduction}

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Despite the progress of diagnosis and treatment of OSCC, the survival index continues to be small. Prevention of cancer using pharmacological, biological, and nutritional interventions is the primary focus of chemoprevention researches \cite{1,2}. Plants are sources of phytochemicals with anticancer potential to interfere with targets implicated in carcinogenesis and in tumor cell biology \cite{3}. Cinnamon, scientifically named \textit{Cinnamomum spp}, is a plant with many uses in herbal medicine. This evergreen shrub belongs to the \textit{Lauraceae} family endemic to Sri Lanka and Southeast regions of India. It contains mucilage, tannin, sugar, resin, limonene, safrole and essential oil that possesses antibacterial, antiseptic, antiviral, and antifungal properties \cite{4}.

Cinnamon bark possessing potent antioxidants comparable to that of synthetic antioxidants with promising potentials to improve oxidative stability of foods \cite{5}. The cinnamon-derived dietary factor cinnamic aldehyde activates the protein expression of Nr2-dependent antioxidant response in human epithelial colon cells \cite{6}. In 2009, Moselhy and Ali \cite{7} evaluated the hepatoprotective effect of cinnamon extracts against carbon tetrachloride induced oxidative stress and liver injury in rats and concluded that this extract can be used as a therapeutic regime in treatment of some hepatic disorders without any side effects.

Cinnamon extract has been demonstrated by Kwon et al. \cite{8} to act as an antimelanoma agent by targeting angiogenesis and the cytolytic effector function of CD8 positive T cells. Koppikar et al. \cite{9} reported that aqueous cinnamon extract (ACE) from the bark of \textit{Cinnamomum cassia} causes apoptosis in human cervical cancer cell line through loss of mitochondrion membrane potential.

Cinnamon polyphenols was found to exert neuroprotective effects in glial cells by the regulation of Bcl-2 family members and enhancing the expression of protein coding gene SIRTI during oxidative stress \cite{10,11}. In 2015, Assadollahi et al. \cite{12} investigated the effects of \textit{Cinnamomum zeylanicum} aqueous extract on the human myelocytic leukemia cell line. The authors concluded that supplemented \textit{Cinnamomum zeylanicum} aqueous extract induced apoptosis in the human myelocytic leukemia cell line. Selective destruction of tumor cells without damaging normal cells is an important goal for cancer chemotherapy in the present century. Therefore, the purpose of this study was to investigate the effect of ACE on 7, 12-Dimethylbenz[a]anthracene (DMBA)-induced
oral carcinogenesis in hamster cheek pouch (HCP) mucosa. The null hypothesis of the present study was that there is no effect of ACE on DMBA-induced oral carcinogenesis.

2. Materials and methods

2.1. Animals’ preparation and grouping

Sixty pathogen free male golden Syrian hamsters were selected. They were 6 weeks old and weighed 150–200 g. They were kept in a light-controlled room; the temperature and relative humidity were kept constant. They received commercial soft diet and water. All experimental procedures were performed under a protocol approved by the ethical committee of Faculty of Dentistry, Mansoura University, Egypt. The hamsters were randomly divided into six equal groups. The hamsters of groups I, II and III received no treatment, DMBA and ACE respectively, for 16 weeks. Groups IV and V were handled as group II and concomitantly treated with ACE for the same period and additionally group V received ACE for other 16 weeks after the stoppage of DMBA application. Group VI hamsters were handled as group III and additionally received DMBA for other 16 weeks after the stoppage of ACE supplementation.

2.2. DMBA application and tumor induction

Left buccal pouches of hamsters were topically treated with 0.5% DMBA (Sigma Chemical Company, St Louis, MO, USA) dissolved in mineral oil (Acros organics, New Jersey, USA) with a camel hair paintbrush 3 times/week [13].

2.3. Aqueous cinnamon extract supplementation

Specimens of Cinnamomum cassia bark were authenticated by the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. The specimens were ground into a fine powder and 50 g of cinnamon powder was dissolved in 500 mL of distilled water and boiled for 3 h. After cooling, simple filtration was applied. Evaporation of water was performed under high vacuum using a rotary evaporator. Freeze-drying was performed to obtain solid powder and radiation was used for sterilization. The administered dose was 500 mg of cinnamon/kg body mass/day through gastric gavage [14].

2.4. Clinical evaluation

Clinical features were observed and recorded during experimental periods as number of died animals, hair loss, skin eruptions and cheek pouch necrosis.

2.5. Biopsy collection

Hamsters of each group were euthanized according to the experimental schedule. The buccal pouches were excised by making incision behind left ears. A blunt forceps was introduced from the mouth to the mental schedule. The buccal pouches were excised by making incision towards the oral cavity. The excised pouches were washed and soaked in neutral formalin then pushed into the pouch from skin the pouch were cleared to free the pouch. A piece of gauze was rolled behind left ears. A blunt forceps was introduced from the mouth to the mental schedule. The excised pouches were excised by making incision 2.5. Biopsy collection

Left buccal pouches of hamsters were topically treated with 0.5% DMBA (Sigma Chemical Company, St Louis, MO, USA) dissolved in mineral oil (Acros organics, New Jersey, USA) with a camel hair paintbrush 3 times/week [13].

2.3. Aqueous cinnamon extract supplementation

Specimens of Cinnamomum cassia bark were authenticated by the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. The specimens were ground into a fine powder and 50 g of cinnamon powder was dissolved in 500 mL of distilled water and boiled for 3 h. After cooling, simple filtration was applied. Evaporation of water was performed under high vacuum using a rotary evaporator. Freeze-drying was performed to obtain solid powder and radiation was used for sterilization. The administered dose was 500 mg of cinnamon/kg body mass/day through gastric gavage [14].

2.4. Clinical evaluation

Clinical features were observed and recorded during experimental periods as number of died animals, hair loss, skin eruptions and cheek pouch necrosis.

2.5. Biopsy collection

Hamsters of each group were euthanized according to the experimental schedule. The buccal pouches were excised by making incision behind left ears. A blunt forceps was introduced from the mouth to the depth of the pouch. With sharp scissors, the fascia and muscles around the pouch were cleared to free the pouch. A piece of gauze was rolled and soaked in neutral formalin then pushed into the pouch from skin side towards the oral cavity. The excised pouches were washed with saline to remove food debris stored into the pouch. Specimens were fixed in 10% neutral buffered formalin for 24 h, then paraffin sections were prepared for routine haematoxylin and eosin (H & E) stain, periodic acid Schiff (PAS) reagent, CD3 and PDGF immunohistochemical reactivity (Table 1).

2.6. Evaluation and scoring of immunohistochemical staining

For CD3 immunoreactivity, double-blinded calibrated examiners performed the microscopic analysis of the HCP mucosa using a light microscope (Olympus, New York, USA). Each examiner had approximately 15 years of experience in investigating biopsy specimens under a light microscope in their practical laboratories. One expert examiner read specimens, and two days later, the same examiner read all the specimens again to evaluate the intraexaminer variability. Then, the same specimens were read by the second examiner to assess the inter-examiner variability (Table 2). The scoring method was as follow:

1. Negative = 100% of cells were negative.
2. Mild = (> 0%- < 10%) of cells were positive.
3. Moderate = (> 10%- < 50%) of cells were positive.
4. Intense = (> 50%) of cells were positive.

Meanwhile, PDGF immunoreactivity was calculated as follows; five different fields of each slide of the HCP mucosa were imaged at 40X magnification using an Olympus digital camera installed on an Olympus microscope with a 1/2X photo adaptor using 40X objective. The resulting images were analyzed on an Intel Core I3-based computer using Video Test Morphology software (Saint Petersburg) with a specific built-in automated object. The analysis provided an estimated quantification of immunohistochemical results.

2.7. Statistical analysis

Data were analyzed using SPSS (Statistical package for social sciences) (Version 17.0; SPSS; Chicago, IL, USA). Variables were presented as mean ± standard deviation (SD). For the analytical statistics, significant differences were tested using univariate or multivariate analyses of variance to compare between more than two groups for the numerical parametric data, followed by post-hoc LSD for multiple comparisons. Independent sample T test was used to test the statistical significance between two different groups. The statistical tests were based on a type 1 error value of 5% (α = 0.05) and on a power of 0.85 sample size.

3. Results

3.1. Clinical observations

Variants of clinical findings were seen after scarification of animals (Table 3). Died animals were not examined.

3.2. Histopathological and histochemical findings

All groups showed dysplastic changes with varying degrees except groups I and III. The DMBA treated pouches in all groups exhibited signs of epithelial dysplasia and microinvasion of lamina propria with malignant epithelial cells. Invasive carcinoma was detected in all animals of groups II, IV, V and VI.

3.2.1. Group I

The pouches of this group revealed normal structural and architectural features of HCP. The epithelium is very thin with 3–5 cells thick keratinized stratified squamous epithelium. The epithelial layers revealed a single layer of cuboidal basal cells, one to two layers of

| Table 1 | Immunohistochemical markers used in the present study. |
|---------|-------------------------------------------------------|
| Antibody | Dilution | Cellular localization | source                   |
| CD3 monoclonal antibody | 1/100 | Cell membrane of T lymphocytes | (Cell Marque, California, USA) |
| PDGF polyclonal antibody | Ready to use | Secreted | (Santa Cruz, Texas, USA) |
polyhedral spinous cells and one to two layers of flattened granular cells with fine keratohyaline granules. The epithelium was covered with thin layer of orthokeratin. The epithelium connective tissue interface was relatively flat with no rete processes. The basement membrane was intact. The submucosa was composed of delicate and loose connective tissue with mild inflammatory infiltrate (Figs. 1A, 2A).

3.2.2. Group II

The DMBA treated pouches exhibited severe dysplastic changes in the form of epithelial hyperplasia with bulbous, broad, and drop shaped rete processes. The observed criteria of epithelial dysplasia were basilar hyperplasia, loss of polarity of basal cells, keratinization of spinous cells, increased nuclear/cyttoplasmic ratio, pleomorphism, and increased mitotic activity with abnormal mitotic figures. The basement membrane showed areas of discontinuity and microtearing. The invasive epithelial cells appeared as epithelial pearls with keratin foci or cell nests without keratin or in the form of detached and scattered cells. Deep invasive carcinomas were recorded in 90% of the samples (Figs. 1B, 2B).

3.2.3. Group III

Sections of this group showed high degree of similarity to group I where the basal cells were arranged in a single row resting on flat and intact basement membrane with no rete ridges. The underlying connective tissue was more or less similar to that of group I (Figs. 1C, 2C).

3.2.4. Group IV

All specimens of this group showed moderate signs of epithelial dysplasia. The pouches of this group revealed discontinuity of basement membrane with invasion to the underlying connective tissue with malignant epithelial cells. Deep invasive carcinomas were recorded only in 60% of the samples while 40% showed no invasion (Figs. 1D, 2D).

3.2.5. Group V

The pouches of this group showed mild to moderate dysplastic changes. Deep invasive carcinomas were recorded only in 50% of the samples while 50% showed no invasion (Figs. 1E, 2E).

3.2.6. Group VI

The epithelium of this group revealed mild dysplastic changes and the basement membrane showed discontinuity and microtearing. Only 40% of pouches showed deep invasive carcinoma and the remaining pouches revealed no invasion (Figs. 1F, 2F).

3.3. Immunohistochemical findings

3.3.1. CD3

The immunohistochemical positive reaction was detected as brown deposits at membrane of lymphocytes. One-way MANOVA test for CD3 revealed an overall significant difference between groups I, II, III, and IV (P < 0.001). Independent sample T test revealed significant differences between groups II & V and between groups III & VI (P < 0.001). LSD post-hoc test revealed significant difference between Groups I & II, I & III, I & IV, II & III, II & IV and III & IV. The mean value for lymphocytes expressing CD3 in group I was 2.95 ± 0.08. The lowest mean number of positive cells was for group III (2.39 ± 0.22). While the highest mean number of positive cells was detected at group II (13.75 ± 0.02). The mean number of inflammatory cells in group IV, V and VI was 3.20 ± 0.03, 4.06 ± 0.02, and 5.45 ± 0.07, respectively (Tables 4-6, Fig. 3).

3.3.2. PDGF-BB

The immunohistochemical positive reaction was detected as brown deposit at the cytoplasm of endothelial cells and fibroblasts and secreted in extracellular matrix. One-way MANOVA test for PDGF-BB revealed an overall significant difference between groups I, II, III, and IV (P < 0.001). Independent sample T test revealed significant differences between groups II & V and between groups III & VI (P < 0.001). LSD post-hoc test revealed significant difference between Groups I & II, I & III, I & IV, II & III, II & IV and III & IV. The mean value for PDGF in group I was 8.61 ± 0.08. The highest reaction for this protein was detected at the epithelium and underlying connective tissue in fibroblasts and endothelial cells of group II (41.46 ± 0.745). Group III

Table 2

| Features | Intraexaminer variability (mean ± SD) | Interexaminer variability (mean ± SD) |
|----------|-------------------------------------|-------------------------------------|
|          | First reading | Second reading | P value | First reading | Second reading | P value |
| Number of cells expressing CD3 | 2.8 ± 0.7 | 2.8 ± 0.9 | 0.600 | 0.3 ± 0.8 | 0.3 ± 0.7 | 0.726 |

Table 3

| Features | Groups |
|----------|--------|
|          | I | II | III | IV | V | VI |
| 1. Necrosis | No | Extensive starts at 2nd week and ends at 3rd week. | No | Extensive starts at 2nd week and ends at 3rd week. | Extensive starts at 4th week and ends at 5th week. | Less extensive starts at 6th week except 2 hamsters developed necrosis at 3rd week and disappeared at 4th week. |
| 2. Hair loss | No | Starts at 2nd week. | No | Starts at 2nd week. | Starts at 5th week. | Starts at 18th week. |
| 3. Skin eruptions | No | 0.5–1 mm in diameter started at 6th week. | No | 0.5–1 mm in diameter. | 0.5–1 mm in diameter. | 0.5–1 mm in diameter. |
| 4. Gross appearance | No | Outward exophytic | No | Papillomatous growth occurred at 6th week. | Exophytic lesion occurred at 10th week. | Outward growth occurred at 24th week. |
| | No | ulcer occurred at 6th week. | No | Large ulcer occurred at 12th week. | Large ulcer occurred at 15th week. | Less extensive ulcer occurred at 30th week. |
| 5. Deaths | No | 2 hamsters were dead at 13th and 15th weeks. | No | No | No | No |
| 6. Body weight | Put on weight. | Sever weight loss. | Put on weight. | Mild weight loss. | Sever weight loss. | Mild weight loss. |

Common clinical findings observed in the studied groups.
revealed the least expression of PDGF-BB in epithelial and connective tissue cells (7.52 ± 0.214). The mean values for groups IV, V and VI were 15.05 ± 0.05, 14.91 ± 0.03 and 12.09 ± 0.08, respectively (Tables 4–6, Fig. 3).

4. Discussion

The Syrian golden hamster is used extensively in biomedical research. Due to its accessibility, the HCP is an excellent model system for the induction of OSCC by chemical carcinogens and is useful for testing chemopreventive agents [15]. It offers unique features useful in studies for vascular physiology, microbiological, immunological, pathological studies and oncology [16].

In the present study, the histological results of group I were in consistent with the results described by Veys et al. [17]. The authors reported that HCP mucosa is a thin stratified keratinized squamous epithelium showing four cell layers and the interface between these layers and the underlying fibrous connective tissue is almost flat. Regarding group II histological results, they were in consistent with the results of Grawish [18] who found that application of DMBA on the HCP mucosa produces SCC that is histologically similar to human oral SCC. Carcinoma is preceded by a sequence of hyperplasia-papilloma/dysplasia-carcinoma, similar to human leukoplakia.

Aqueous cinnamon extract was used in the present study avoiding its topically applied adverse effects such that associated with cinnamon-flavored toothpaste or chewing gum. It had been reported that such products induce orofacial granulomatosis, oral mucosal reactions, burning sensations, red or white lesions and have even been implicated in rare cases of leukoplakia and carcinoma [19–26]. Administration of 500 mg of cinnamon/ kg body mass/day for 16 weeks in group III didn’t produce any side effects and this consistent with dose used by Lopes et al. [27] who reported that Cinnamon extract improves the body composition and attenuates lipogenic processes in the liver and adipose tissue of rats.

The HCP mucosa of groups IV, V and VI showed moderate to mild dysplastic changes in the stratified squamous epithelium and underlying connective tissue. Group VI results was the best followed by group V and the worst one was group IV results. This means that ACE has the potentiality preventing oral cancer initiation better than inhibiting oral cancer progression. These results are consistent with the findings of Chang et al. [28] who evaluated the anti-cancer activities of *Cinnamomum cassia* essential oil and its major constituent, cinnamaldehyde, in human oral squamous cell carcinoma HSC-3 cells. They suggested that *Cinnamomum cassia* essential oil and cinnamaldehyde may possess anti-oral cancer activity in HSC-3 cells. In addition, Schoene et al. [29] suggested that the potential of ACE to interact with phosphorylation/dephosphorylation signaling activities to reduce cellular proliferation in tandem with a block at the G2/M phase of the cell cycle. Moreover, Schoene et al. [30] found that ACE significantly modulated two signaling proteins, p38 MAPK and cyclin B, that regulate progression...
through G2/M.

In addition to PDGF role in autocrine growth stimulation of tumor cells, PDGF has also been suggested to regulate tumor stroma fibroblasts and tumor angiogenesis [31]. The HCP mucosa of groups I and III showed few epithelial cells stained positively for PDGF. In OSCC, positive staining was increased in groups II. ACE inhibited the activation of PDGF in group VI and group V more than group IV. Yu et al. [32] demonstrated that PDGFs and their receptors are not only involved in human cancers through a paracrine but also an autocrine stimulation of tumor cell growth. It has a mitogenic effect on tumor cells and also nourishes the tumor proliferation by stimulating the angiogenetic process directly and indirectly [33,34].

Table 4
One way-MANOVA and LSD post-hoc test for CD3 and PDGF for all groups.

| Groups | Pillai's Trace (F ratio and P value) | LSD post-hoc (Mean ± SD) |
|--------|----------------------------------|-------------------------|
|        | CD3 (11619.131, 0.001)            | CD3                     |
|        | PDGF (18592.247, 0.001)           | I : 2.95 ± 0.08*        |
|        |                                  | II : 13.75 ± 0.22*      |
|        |                                  | III : 4.06 ± 0.02       |
|        |                                  | IV : 3.20 ± 0.03*       |
|        |                                  |                         |

Table 5
Mean and SD for group II and V regarding values of PDGF-BB and CD3.

| Marker | Group | Mean ± SD | t    | df | P value |
|--------|-------|-----------|------|----|---------|
| PDGF   | II    | 41.46 ± 0.74 | 76.678 | 18 | 0.001   |
|        | V     | 14.91 ± 0.03  |       |    |         |
| CD3    | II    | 13.75 ± 0.22  | 28.323 | 18 | 0.001   |
|        | V     | 4.06 ± 0.02   |       |    |         |

In patients with head and neck cancer, compromised antitumor functions of T lymphocytes have been observed [35]. Especially, tumor-infiltrating lymphocytes contribute to local tumor suppression as well...
5. Conclusion

On the basis of this research and within its limitation, the use of ACE has a beneficial role in preventing cancer initiation better than inhibiting cancer progression. More research is needed to confirm these results and to prove its efficacy against cancer especially; it is already clear that ACE is a natural food additive that has proven benefits for optimum health and wellness.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbr.2017.08.014.

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Table 6

Mean and SD for group III and VI regarding values of PDGF-BB and CD3.

| Marker | Group | Mean ± SD | t | df | P value |
|--------|-------|-----------|---|----|---------|
| PDGF   | III   | 7.52 ± 0.21 | −179.514 | 18 | 0.001   |
|        | VI    | 12.09 ± 0.08 | 143.474 | 18 | 0.001   |
| CD3    | III   | 2.39 ± 0.02 | 14.012  | 18 | 0.001   |
|        | VI    | 5.45 ± 0.07 | 7.567   | 18 | 0.001   |

Fig. 3. Bar chart for mean values of CD3 and PDGF for groups I, II, III and IV.

Table 6

Mean and SD for group III and VI regarding values of PDGF-BB and CD3.
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