INDUCTION OF APOPTOSIS BY PIPER BETEL LEAF EXTRACT ON 7, 12 DIMETHYLBENZ [A] ANTHRACENE INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

SELVAMUTHUKUMAR, S.1,2, PUSHPALATHA, R.2, SETHUPATHY, S.3, MANAVALAN, R.1 AND KARAR, P. K.1

1Department of Pharmacy, Annamalai University, Annamalai Nagar 608002, 2Formulation Development, Shasun Chemicals and Drugs Limited, Periyakalapet, Pondicherry 605014, 3Department of Biochemistry, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar 608002, India. E mail: smk1976@gmail.com

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Abstract: The induction of apoptosis by Piper betel leaf extracts during 7, 12 dimethylbenz [a] anthracene (DMBA) induced hamster buccal pouch (HBP) carcinoma was studied in male Syrian hamsters using DNA fragmentation and the apoptosis-associated proteins, tissue transglutaminase (tTG) and Bcl-2. Hamsters were divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of DMBA in liquid paraffin on the right buccal pouches three times a week for 14 weeks. Group 2 animals painted with DMBA as in group 1, in addition received 500 mg/kg body weight chloroform extract of Piper betel leaves orally on days alternate to DMBA application. Group 3 animals painted with DMBA as in group 1, in addition received 500 mg/kg body weight methonolic extract of Piper betel leaves orally on days alternate to DMBA application. Group 4 animals received neither DMBA nor betel leaf extracts and served as the control. The experiment was terminated at the end of 14 weeks. Administration of Piper betel leaf extracts (500 mg/kg body weight) to animals painted with DMBA inhibited DMBA-induced oral cancer as revealed by the absence of neoplasm, induction of tTG and inhibition of Bcl-2 expression. The results of the present study suggest that Piper betel leaves may exert its chemopreventive effect by inducing apoptosis.

Key words: Apoptosis, Piper betel, Carcinoma, Chemoprevention

INTRODUCTION

In 1972 Kerr, Wyllie and Currie distinguished between two forms of cell death, necrosis and apoptosis [1]. Cancer development is known to be associated with increased cell proliferation and decreased apoptosis [2]. Induction of apoptosis serves as a means to suppress or reverse cancer development. Agents capable of inducing apoptosis are recognized as chemopreventive agents [3]. Radiation and the anticancer agent tamoxifen, which possess apoptosis-inducing properties have been used to treat cancer [4,5]. Medicinal plants and natural dietary constituents such as curcumin, resveratrol, genistein and flavone have been reported to induce apoptosis in malignant cells in vitro [6-10].

Piper betel Linn. (Piperaceae) is a perennial dioecious crop that is cultivated in the hotter and damper parts of India [11]. It is popularly known as “vettilai” in Tamil, “Betel,” “Betel leaf vine,” and “Betel pepper” in English and “Tambulavalli” in Sanskrit [12]. Fresh leaves are chewed with betel nut and other adjuncts in most parts of India and other countries of East Asia. The leaves contain aromatic, digestive, and carminative properties [13]. The leaves of P. betel are one of the most promising species for cancer chemoprevention [14], because betel leaf extract perhaps contains some tumor-inhibitory principles [15]. On account of these properties, the
present experiment was set up to look at its chemopreventive effect in oral cancer.

**MATERIALS AND METHODS**

**Plant material:** *Piper betel* was purchased in the local market, Chidambaram, Cuddalore district, Tamil Nadu, India. The herbarium of this plant was identified and authenticated (Herbarium No. AU 3898) by the taxonomist, Department of Botany, Annamalai University, Tamil Nadu.

**Preparation of extract:** Fresh leaves were air-dried in shade at room temperature. The dried leaves were coarsely powdered mechanically; the powdered leaves were extracted successively with petroleum ether, chloroform and methanol by maceration.

**Experimental animals:** All the experiments were carried out with male Syrian hamsters aged 8 to 10 weeks, weighing 85-90 g, obtained from the Central Animal House of University. The animals were housed in polypropylene cages (6 animals/cage). They were maintained under controlled conditions of temperature and humidity with an alternating 12-h light/dark cycle. All animals were fed standard pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*.

**Treatment Schedule:** The animals were randomized into experimental and control groups and divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of 7,12 dimethylbenz[a]anthracene (DMBA) (Sigma, USA) in liquid paraffin on the right buccal pouches using a number 4 brush three times a week for 14 weeks. Each application leaves approximately 0.4 mg DMBA [16]. Group 2 animals were painted with DMBA as in group 1. In addition, the animals were administered chloroform extract of *P. betel* leaves at a dose of 500 mg/kg body weight orally on days alternate to DMBA application. Animals in group 3 animals were painted with DMBA as in group 1. In addition, the animals were administered methanol extract of *P. betel* leaves at a dose of 500 mg/kg body weight orally on days alternate to DMBA application. Group 4 (untreated control) animals received neither DMBA nor *P. betel*. The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. At sacrifice, the buccal pouch was excised, grossly examined, and a portion of the tissue was used for histopathological examinations. The remaining tissues were immediately stored at 70°C until use. Studies were carried out in buccal pouch tumour tissues in animals that developed tumours and in normal pouch tissues in animals that did not develop tumours.

**Extraction of low molecular weight DNA:** Low molecular weight DNA was extracted by the method of Gong et al. [17]. To the tissues (~50–100 mg), 40 ml of phosphate-citrate buffer (192 parts of 0.2 M Na2HPO4 and eight parts of 0.1 M citric acid, pH 7.8) was added and kept at room temperature for 30 min. After centrifugation at 1000×g for 5 min, the supernatant, 3 ml of 0.25% Nonidet NP-40 in distilled water was added followed by 3 ml of RNase A (1 mg/ml in water). After 30 min incubation at 37°C, 3 ml solution of proteinase K (1 mg/ml) was added and incubated for a further period of 30 min at 37°C. The amount of DNA was quantified from its absorbance at 260 nm using a spectrophotometer.

**DNA gel electrophoresis:** Equal quantities of DNA samples were mixed with an appropriate volume of 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) and electrophoresed through a 1% agarose gel in 0.5× TBE buffer (45 mM Tris–borate and 1 mM EDTA). DNA in the gels was visualised by adding 0.5 mg/ml of ethidium bromide to the electrode buffer.

**SDS-PAGE and Western blot analysis:** Approximately, 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The protein concentrations of the lysates were determined spectrophotometrically. Equivalent protein extracts (60 mg) from each sample were electrophoresed on 10% SDS-PAGE gels and electrophoretically transferred on to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA, USA). The blot was treated for 1 h with blocking solution containing 3% BSA in Tris buffered saline (TBS) containing 150 mM NaCl and 50 mM Tris pH 7.4 at room temperature. The blot was rinsed once with TBS and incubated with 1:1000 dilution of anti-Bcl-2 (Santa Cruz Biotechnology, CA, USA) and anti-tTG (Neomarker, USA) for 2 h. The blot was washed with TBS-T (TBS with 0.1% Tween 20) four times for 10 min each. tTG and Bcl-2 were detected by incubating corresponding horseradish conjugated
secondary antibodies (1:4000 diluted) to tTG and Bcl-2 for 30-45 min at room temperature. After four washes (10-min. each) in TBS-T, the transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham, UK). Densitometry was performed on a Microtek scan maker IIISP flat bed scanner and quantified with Total Lab v2.01 software.

Statistical analysis: Statistical analysis for densitometric analysis was carried out using analysis of variance followed by Duncan’s Multiple Range Test (DMRT). Treatment mean differences with P<0.05 were considered statistically significant.

RESULTS

Table 1 summarizes the incidence of oral preneoplastic lesions and neoplasms in different groups. Exophytic tumours induced by DMBA in the oral cavity of hamsters in group 1 were well-differentiated squamous cell carcinomas. The incidence of oral neoplasms in group 1 was 100%, whereas in group 2, only moderate keratosis, mild hyperplasia and mild inflammatory exudate were observed. No malignant neoplasms or preneoplastic lesions were observed in animals in groups 3 and 4.

Figure 1 shows the agarose gel electrophoresis of DNA extracted from buccal pouch mucosa of control and experimental animals. Internucleosomal DNA fragmentations and a profile typical for apoptotic cells were not observed in any of the groups (Group 1 to group 4). The effect of *P. betel* leaves on tTG protein expression in the buccal pouch mucosa of control and experimental animals is shown in figure 2. The mean tTG expression from control lysates was 131, 435 arbitrary units and was designated as 100% in the graph. Each bar represents the mean tTG expression ±S.D. of six determinations per treatment performed after 14 weeks. Induction of tTG was observed following administration of *P. betel* leaf extract to animals painted with DMBA by 152% relative to control. A representative immunoblot is shown in figure 3.

Figure 4 shows the effect of *P. betel* on Bcl-2 protein expression in the buccal pouch mucosa of control and experimental animals. The mean Bcl-2 expression from control lysates was 119, 604 arbitrary units and...
tTG activity has been reported to be low in human prostate adenocarcinoma and N-ethyl-N-nitrosourea induced rat brain carcinogenesis [18,19]. Overexpression of Bcl-2, a key event in cancer development, progression and resistance to treatments has been demonstrated in several malignancies including cancer of the oral cavity [20,21]. Although we found enhanced expression of Bcl-2 in DMBA-induced HBP tumours, the activity of tTG was higher than in controls. HBP carcinogenesis, a stepwise process involves development of leukoplakia at 6-8 weeks, carcinoma in situ at 8-10 weeks, epidermoid carcinomas at 10-12 weeks and exophytic, invasive tumours at 12-14 weeks, followed by tumour necrosis and death of the animals between 16-20 weeks [16]. Sequential variations in tTG activity could occur, with lowest levels in the early active proliferative phase of HBP carcinogenesis and subsequent rise in later stages as

was designated as 100% in the graph. Each bar represents the mean Bcl-2 expression ± S.D. of six determinations per treatment performed after 14 weeks. Administration of chloroform extract and methanolic extract of *P. betel* to animals painted with DMBA down regulated Bcl-2 protein expression by 64.7% and 72.5% respectively as compared to control. A representative immunoblot is shown in figure 5.

**DISCUSSION**

Fig. 3: Western blot analysis of buccal pouch extract probed with anti-tTG antibody. Lane 1: control; lane 2: DMBA; lane 3: DMBA + chloroform extract; lane 4: methanol extract

Fig. 5: Western blot analysis of buccal pouch extract probed with anti-Bcl-2 antibody. Lane 1: control; lane 2: DMBA; lane 3: DMBA + chloroform extract; lane 4: methanol extract

Fig. 4: Densitometric analysis of Bcl – 2 protein expression. Values not sharing a common letter (a–d) differ (P<0.05).
necrosis sets in. Further studies are required to understand tTG expression in DMBA induced HBP tumours. We observed induction of apoptosis following administration of P betel extract to animals painted with DMBA as revealed by the induction of tTG and down regulation of Bcl-2 protein expression. However, P betel had no effect on internucleosomal DNA fragmentation.

The onset of apoptosis in vivo is often characterized by induction of the tTG gene [22]. tTG induction/activation results in excessive cross-linking of cytoplasmic and membrane proteins preventing leakage of intracellular components. This caging effect of tTG is implicated in the apoptotic process [23,24]. Substances such as 4-hydroxyphenylre- tinamide, butyrate and propionate are known to induce apoptosis in carcinoma cell lines by inducing tTG [25,26]. The activity of tTG was found to be increased when cells cease to proliferate [27]. Induction of tTG by P. betel further validates its antiproliferative effects. Diminished expression of Bcl-2 has been observed in cell types undergoing apoptosis [6,7,28]. Naturally occurring substances such as hibiscus protocatechuic acid, curcumin, resveratrol and garlic have been reported to induce apoptosis in carcinoma cell lines by decreasing Bcl-2 expression [6,7,29]. The decrease in Bcl-2 protein expression in pouch mucosa of animals administered P. betel following DMBA treatment correlates with its apoptosis-inducing effects. Induction of tTG is known to be associated with Bcl-2 down regulation [30]. Our results substantiate these findings. DNA fragmentation is believed to be a hallmark of apoptosis. Several studies have demonstrated a positive association between DNA fragmentation and apoptosis [8,31, 32]. The absence of internucleosomal DNA fragmentation in DMBA-treated animals reflects the prevention of apoptotic mechanisms in malignant cells. We did not observe internucleosomal DNA fragmentation during chemoprevention of HBP carcinogenesis by P. betel. Our results support the finding that key changes in cell death can occur without DNA fragmentation, but the later alone should not be considered as a criterion for assessing apoptotic cell death [33,34]. Betel leaf extract as well as its constituents β-carotene and α-tocopherol have been reported for cancer chemoprevention. [14,15,35,36]. Our results clearly demonstrate that P. betel extracts induces apoptosis by inducing tTG and inhibiting Bcl-2 protein expression without causing DNA fragmentation. We feel that apoptosis induction by P. betel extracts represents one of the possible mechanisms that could account for its antineoplastic and antiproliferative activities. These results may provide new insights into the molecular mechanisms of apoptosis and chemopreventive effects of P. betel and its constituents.

Abbreviations used: HBP- Hamster buccal pouch, tTG- tissue Trasglutaminase, Bcl-2- B-cell CLL/lymphoma 2, DMBA- 7,12-dimethyl benz[a]anthracene, TBE- Tris Borate EDTA, SDS-PAGE – Sodium dodecyl sulphate Poly acrylamide gel electrophoresis, BSA – Bovine serum albumin

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