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Chemoprevention by Quercetin of Oral Squamous Cell Carcinoma by Suppression of the NF-κB Signaling Pathway in DMBA-treated Hamsters

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Abstract. *Background/Aim: The aim of this study was to investigate the effects of the flavonoid quercetin on chemoprevention of oral squamous cell carcinoma (OSCC). The study involved molecular signaling pathways in 7,12-dimethylbenz(a) anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. Materials and Methods: DMBA (0.5%) was painted at the right buccal pouches of hamsters for 14 weeks to induce carcinoma. DMBA-treated hamsters received simultaneous doses of quercetin. Animals without DMBA induction were used as normal controls. The incidence of OSCC and the severity of pre-malignant lesions were determined histologically. Apoptosis in the pouch tissue was determined by TUNEL staining. The mRNA and protein expression of NF-κB p50 and p65, as well as Bcl-2 and Bax genes were analyzed using RT-PCR and Western blotting, respectively. Results: Quercetin, at various doses, significantly reduced OSCC incidence and severity of hyperplasia and dysplasia compared to the DMBA-induction-only group (p<0.01). Apoptosis was induced by quercetin treatment compared to the DMBA-induction-only group (p<0.01). mRNA and protein expression of NF-κB p50, p65 as well as Bcl-2 genes were significantly suppressed by quercetin at high doses compared to DMBA induction only (p<0.05). However, mRNA and protein expression of the Bax gene was increased by quercetin treatment at medium and high doses, compared to the DMBA-induction-only group (p<0.05). Quercetin significantly reduced body-weight loss compared to the DMBA-induction-only group (p<0.05). Conclusion: Quercetin reduced tumor incidence and induced apoptosis through modulation of NF-κB signaling and its target genes Bcl-2 and Bax in the DMBA-induced carcigenesis hamster model, suggesting the potential of quercetin as a candidate for OSCC chemoprevention.*

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Surgery and chemotherapy for oral squamous cell carcinoma (OSCC) have limited efficacy (1-3). Thus, there is an urgent need to elucidate the mechanisms of development of OSCC and develop a new strategy for its treatment and prevention.

Quercetin is a flavonoid, present in many fruits and vegetables (4, 5), that can inhibit proliferation and induce apoptosis and arrest many types of human cancer cells (6-10), suggesting its potential for cancer prevention and treatment. Quercetin affects several signal-transduction pathways associated with carcinogenesis including suppression of nuclear factor-kappa B (NF-κB) activation (11) and change of the ratio of pro-apoptotic Bcl-2-associated X protein (Bax) and anti-apoptotic B-cell lymphoma-2 (Bcl-2) proteins (12-14).

Previous reports have suggested anti-cancer efficacy of quercetin on OSCC (15, 16), yet the mechanism of quercetin action remains poorly understood. Therefore, in the current study, we aimed to evaluate the chemopreventive efficacy of quercetin on 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis and

investigate the molecular-signaling pathways of quercetin-induced cell apoptosis in OSCC.

Materials and Methods

Hamsters. Male Syrian hamsters aged 8-10 weeks weighing between 100 and 110 g were obtained from Vital River Laboratories Company, Beijing, China. All animals were fed with an autoclaved laboratory rodent diet. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12-h light/dark cycle in accordance with the regulations of the ethics committee and the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China (OB1511).

Induction of HBP carcinogenesis and treatment. Hamsters were randomized and divided into six groups of 8 animals each. Group 1 served as the normal control without any treatment. Group 2 received quercetin treatment-only, at a dose of 50 mg/kg daily. Groups 3-6 were given DMBA for induction of HBP carcinogenesis. DMBA (0.5%) (Sigma Chemical Company, St. Louis, MO, USA) in liquid paraffin was painted on the right buccal pouches of hamsters with a number-4 brush, three times per week for 14 weeks (17). Group 3 served as the model control and received DMBA only. Group 4, 5 and 6 received simultaneous treatment of quercetin (Sigma Chemical Company, St. Louis, MO, USA) at doses of 12.5 mg/kg (L), 25 mg/kg (M), or 50 mg/kg (H), respectively. Quercetin was administrated *via* oral gavage daily for 14 weeks. Animal body weights were recorded weekly during the experimental period. The experiment was terminated at 14 weeks and all animals were sacrificed. The right pouch of the animal was grossly inspected to evaluate pre-malignant lesions or tumor development and photographed. The buccal pouch tissues of all animals were collected for further analysis.

Histo-pathological analysis. The pouch tissue was fixed in 10% formalin, dehydrated, paraffin embedded, processed and sliced into 5 μm-thick sections. Each sample was cut into 30 sections. The first, 15th, and final sections were stained with haematoxylin and eosin (H&E) for histopathological analysis. Basal-cell hyperplasia, dysplasia and OSCC were diagnosed by a pathologist who was blinded to the experimental groups, according to the established criteria (18).

Apoptosis analysis. Apoptosis in the pouch tissue was determined by TUNEL staining, using a commercially-available kit (In Situ Cell Death Detection Kit, POD; Roche, Penzburg, Germany). The pouch tissue sections were deparaffinized and dehydrated according to standard protocols. Tissue sections were incubated with Proteinase K working solution for 30 min at 21-37˚C. The slides were then washed with PBS (pH 7.2-7.6) twice. The positive control was incubated with DNase I for 10 min at 15-25˚C. The negative control was incubated with labeling solution (without terminal transferase) instead of the TUNEL reaction mixture. The slides were then washed with PBS (pH 7.2-7.6) three times. Converter-POD was added on the slides and incubated in a humidified chamber for 30 min at 37˚C. The slides were then washed with PBS (pH 7.2-7.6) three times. The DAB substrate was added to the slides, which were incubated for 10 min at 15-25˚C. The slides were then washed with PBS (pH 7.2-7.6) three times. The slides were mounted and analyzed by microscopy (Olympus, Melville, NY, USA). The slides were viewed at 400× magnification and apoptotic cells were recognized by the appearance of brown-stained cells. Expression levels were quantified by the average optical density (AOD) of the positive cells in 5 fields/sample with Image-Pro Plus 6.0 software.

Real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from pouch tissue samples in 1 ml of TRIzol reagent (Invitrogen Life Tech, Carlsbad, CA, USA). Then total RNA was treated with DNaseI and subjected to quantitative PCR, which was performed with an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I dye. The primers used for PCR reactions were GAPDH (sense 5'-GGGTGTGAACCATGACAAGT-3'; antisense 5'-AGTGGATGCAG GGATGATGT-3'), Bax (sense 5'-TCATGAAGACAGGGGCCTTT-3'; antisense 5'-CTGTCCAGCTCATCTCCGAT-3'), Bcl-2 (sense 5'-CCTGGCATCTTCTCCTTCCA-3'; antisense 5'-CTGACTGGACAT CTCTGCGA-3'), NFκB-p50 (sense 5'-AAAATATCCACCTGCA CGCC-3'; antisense 5'-CCAGGATTGTAGCCCCGTAT-3') and NFκB-p65 (sense 5'-ACAGATACCACCAAGACGCA-3'; antisense 5'-AGGTCTCGCTTCTTCACACA-3'). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting. Protein from pouch tissue was extracted with RIPA buffer (Beyotime BioTech, Shanghai, China). Protein concentration was determined with the Bradford protein assay kit (KeyGen BioTech). Total proteins were separated by sodium salt-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated with 5% non-fat milk solution for blocking non-specific binding and then with primary antibodies to NF-κB p50 (Ab7971; 1:400); NF-κB p65 (sc-372; 1:500); Bcl-2 (sc-492; 1:1,000); Bax (sc-493; 1:1,000). All antibodies were from Santa Cruz Biotechnology Inc., except anti-NF-κB p50, which was from ABcam, Shanghai, China. After washing twice with tris-buffered saline, membranes were incubated with an appropriate secondary antibody (1:5,000) conjugated with HRP for 2 h at room temperature. Analysis by electro-chemiluminescence was performed according to the manufacturer's instructions using a Bio-Rad imaging system. Quantity One version software (Bio-Rad, Hercules, CA, USA) was used to quantify the density of bands.

Statistical analysis. The data are expressed as mean ± SD. Statistical analysis for body weight was with the Student's *t*-test, while tumor incidence was compared by χ^2-test. The data for densitometric analysis were analyzed using ANOVA followed by Bonferroni post hoc tests. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Quercetin inhibited DMBA induced carcinogenesis. As shown in Figure 1, at the end of the study, tumors developed in the right buccal pouch of hamsters after 14 weeks induction with DMBA. The incidence of OSCC and the severity of premalignant lesions were determined by histological assessment. The animals without DMBA

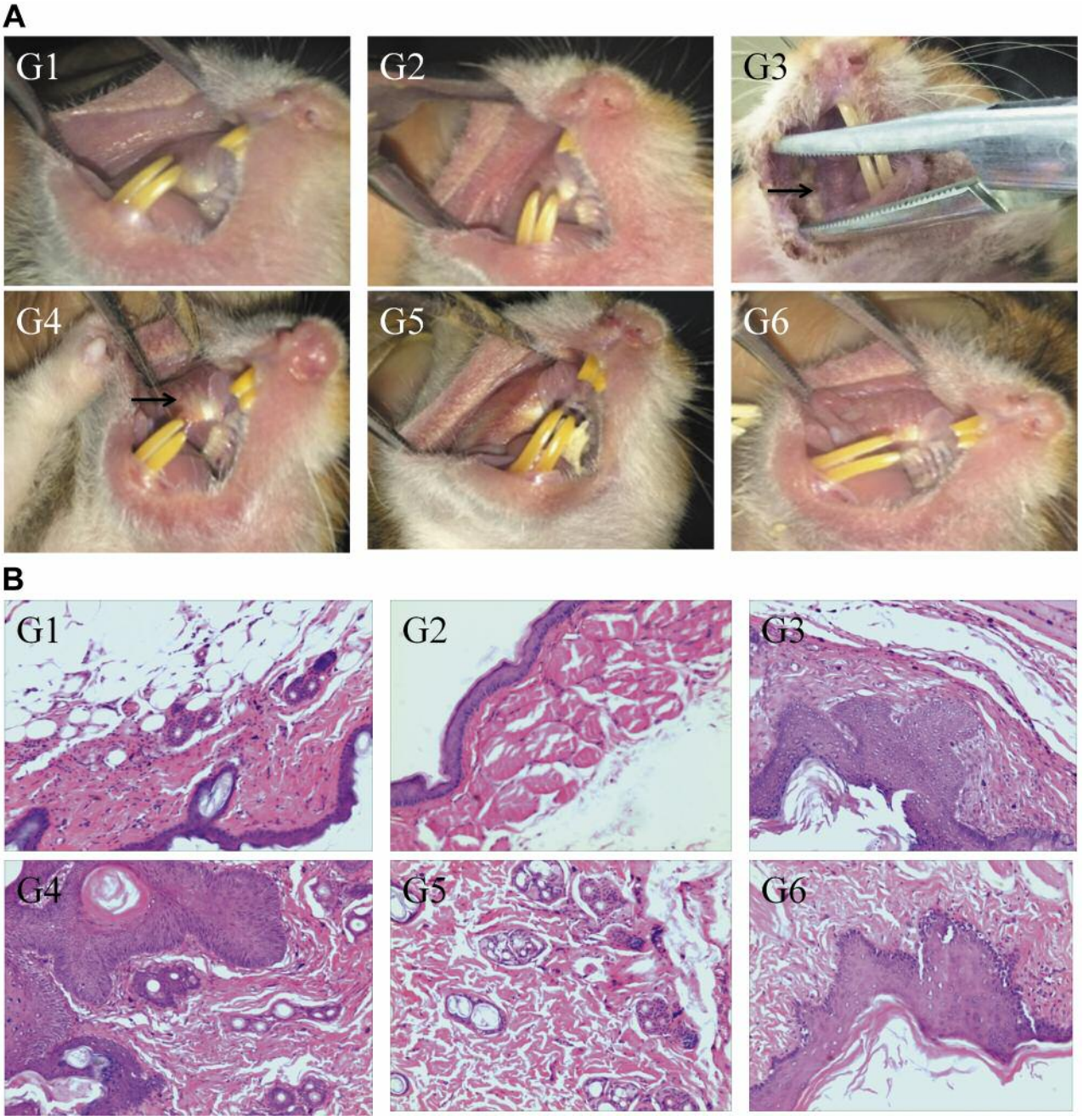

Figure 1. *Effect of quercetin on tumor incidence in DMBA-induced hamster buccal pouch (HBP) carcinogenesis. A. Representative images of the HBP in each group. Black arrows indicate tumors. B. Representative histological images of H&E staining of HBP in each group, normal histology in G1 and G2, well differentiated OSCC in G3 and G4, mild hyperplasia in G5 and G6, (100× magnification).*

induction (Groups 1 and 2) showed normal, intact, and continuous epithelium. However, the animals treated with DMBA (Group 3) showed 100% OSCC incidence and most severe hyperplasia and dysplasia (Figure 1B). Simultaneous treatment with quercetin and DMBA in Group 4, 5 and 6 significantly reduced OSCC incidence and severity of two premalignant lesions as compared to Group 3 ($p<0.01$). No tumor development was found in the animals treated with medium-(Group 5) and high-(Group 6) doses of quercetin, although histopathological assessment of pouch tissue revealed mild to moderate hyperplasia or dysplasia (Table I).

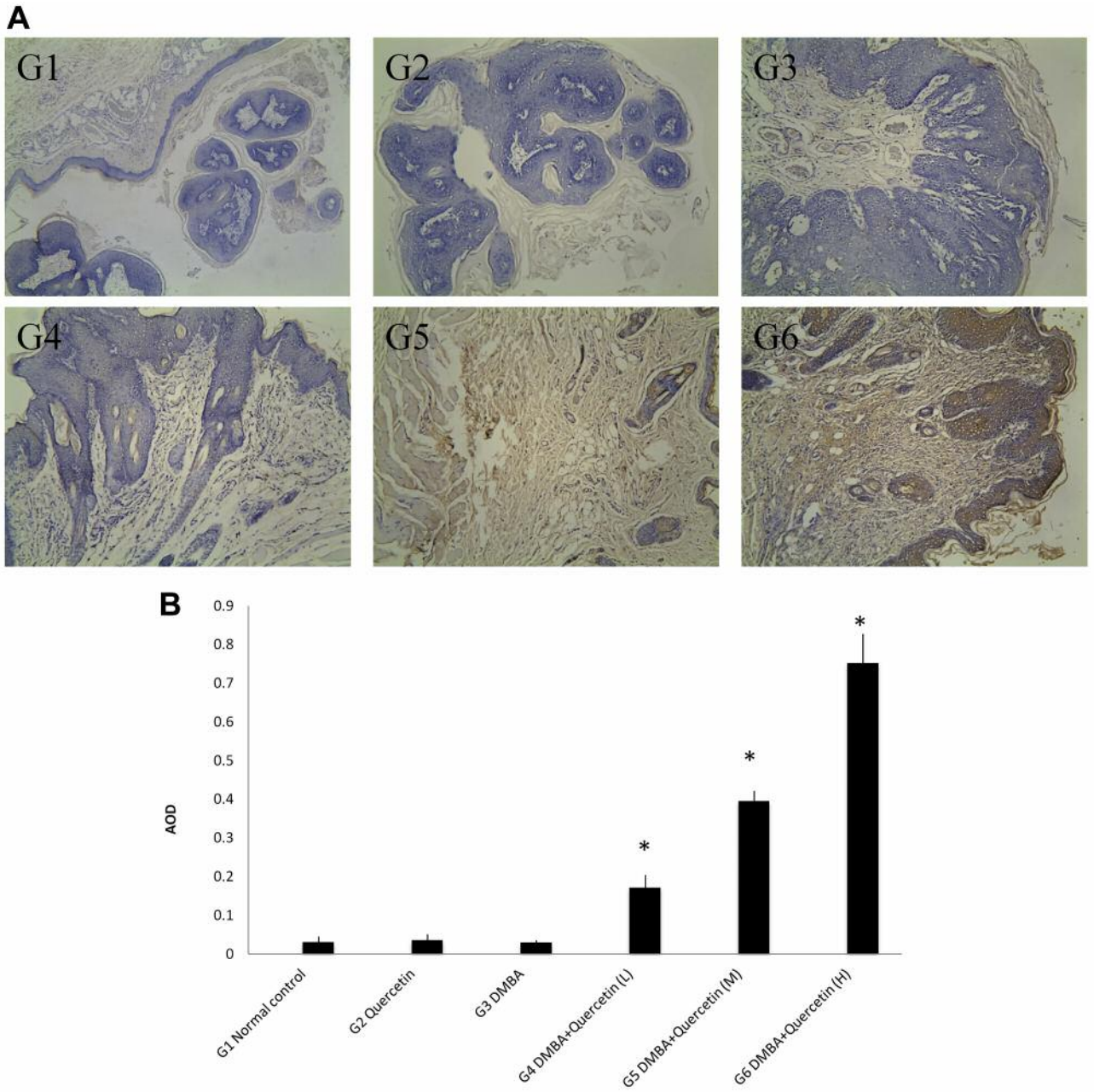

Figure 2. *Effect of quercetin on tumor apoptosis in DMBA-induced HBP carcinogenesis. A. Representative TUNEL images for apoptosis in each group (100× magnification). B. Quantitation of apoptosis by average optical density (AOD). *p<0.01, compared to G3 DMBA.*

Quercetin induces apoptosis in DMBA-induced carcinogenesis. The effect of quercetin treatment on tumor apoptosis was analyzed with TUNEL staining. Representative fields of view from each group are shown in Figure 2A. Simultaneous quercetin treatment along with DMBA in Groups 4, 5 and 6 significantly induced cancer-cell apoptosis in a dose-dependent manner in the DMBA treated animals as compared to Group 3 ($p<0.01$). No significant apoptosis in the quercetin-treated normal animal was found, indicating quercetin can specifically induce cancer-cell apoptosis only (Figure 2B).

Quercetin reduced the expression of NF-κB p50 and p65 in DMBA-induced carcinogenesis. To investigate the effects of quercetin on the NF-κB signaling pathway, we analyzed the mRNA and protein expression of NF-κB family members NF-κB p50 and p65 using RT-PCR and Western blotting,

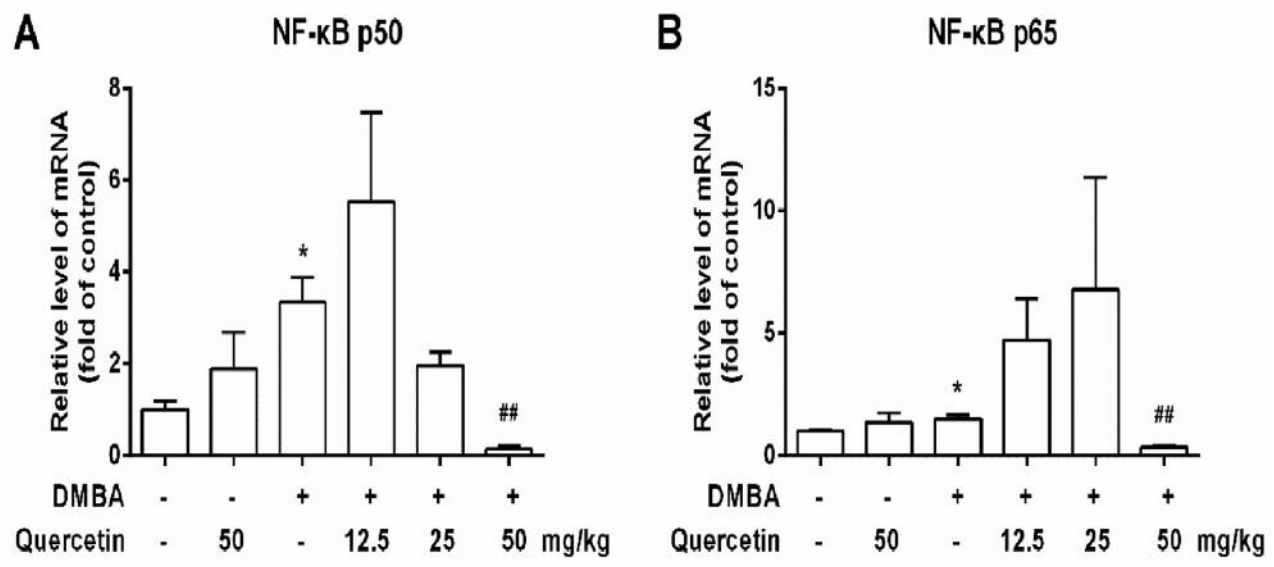

Figure 3. *Effect of quercetin on mRNA expression of NF-κB p50 and p65 in DMBA-induced HBP carcinogenesis. A. NF-κB p50 mRNA expression in each group. B. NF-κB p65 mRNA expression in each group. *p<0.05, when compared to the G1 normal-control group. #p<0.05, when compared with G3, DMBA induction only.*

Table I. *Incidence of OSCC and severity of premalignant lesions in each group.*

Group	Treatment	OSCC incidence (%)	Hyperplasia	Dysplasia
1	Normal control	–	–	–
2	Quercetin	–	–	–
3	DMBA	8/8 (100%)	+++	+++
4	DMBA+ Quercetin (L)	3/8 (37.5%)	++	++
5	DMBA+ Quercetin (M)	–	+	+
6	DMBA+ Quercetin (H)	–	+	

respectively. As shown in Figures 3 and 4, DMBA induction in Group 3 resulted in increased mRNA and protein expression of NF-κB p50 and p65 as compared to the normal-control group ($p<0.05$ and $p<0.01$, respectively). Such increased mRNA expression of NF-κB p50 and p65 were significantly suppressed by simultaneous quercetin treatment at high dose in Group 6 compared to Group 3 ($p<0.01$). Significantly inhibited protein expression of NF-κB p50 and p65 was found in animal groups treated with medium- and high-dose quercetin as compared to Group 3 ($p<0.05$ and $p<0.01$, respectively). Quercetin treatment in the normal animals (Group 2) did not affect mRNA and protein expression of NF-κB p50 and p65 compared to the normal-control group ($p>0.05$).

Effect of quercetin on the expression of NF-κB target genes. Since NF-κB signaling activation can promote the transactivation of genes involved in apoptosis, we further investigated the effect of quercetin on Bcl-2 and Bax expression. As shown in Figures 5 and 6, DMBA induction in Group 3 resulted in increased mRNA and protein expression of Bcl-2 compared to the normal control group ($p<0.05$ and $p<0.01$, respectively). Increased Bcl-2 expression was significantly suppressed by simultaneous quercetin treatment at high dose in Group 6 as compared to Group 3 ($p<0.05$). In addition, DMBA induction in Group 3 resulted in decreased mRNA and protein expression of Bax as compared to the normal control group ($p<0.05$ and $p<0.01$, respectively). Significantly up-regulated mRNA and protein expression of Bax were found in animal groups treated with medium- and high-quercetin dose in a dose-dependent manner compared to Group 3 ($p<0.05$ and $p<0.01$, respectively). Quercetin treatment in the normal animals (Group 2) did not affect mRNA and protein expression of Bcl-2 and Bax as compared to the normal control group ($p>0.05$).

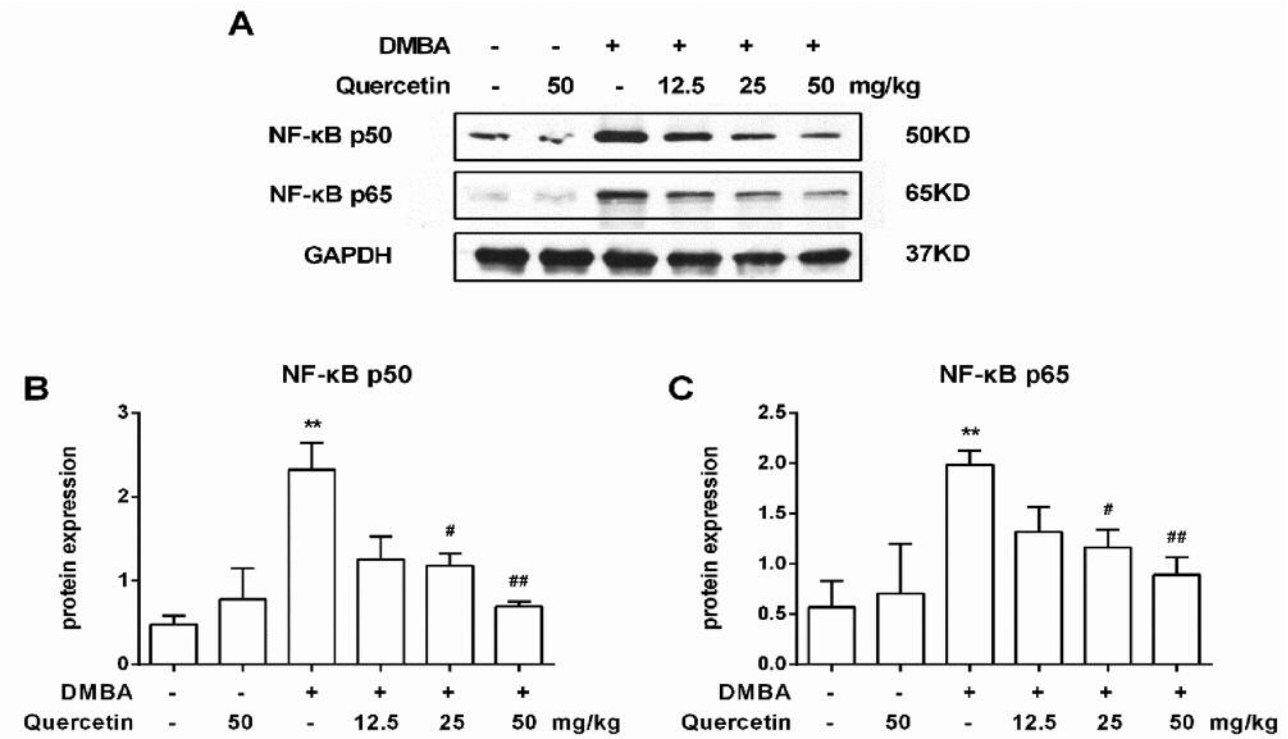

Figure 4. *Effect of quercetin on protein expression of NF-κB p50 and p65 in DMBA-induced HBP carcinogenesis. A. Representative immunoblots of NF-κB p50 and p65 proteins in each group. B. NF-κB p50 protein expression in each group. C. NF-κB p65 protein expression in each group. **p<0.01, when compared to the G1 normal control group. #p<0.05 and ##p<0.01, when compared to G3, DMBA induction only.*

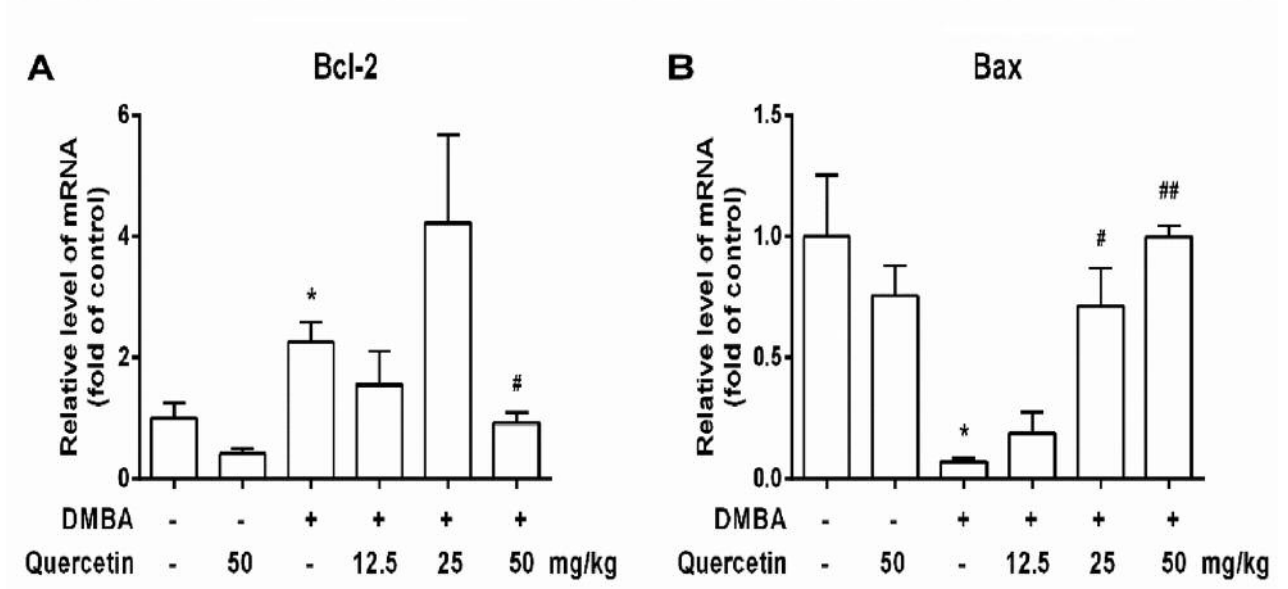

Figure 5. *Effect of quercetin on mRNA expression of Bcl-2 and Bax in DMBA-induced HBP carcinogenesis. A. Bcl-2 mRNA expression in each group. B. Bax mRNA expression in each group. *p<0.05, compared to the G1 normal control group. #p<0.05 and ##p<0.01, compared to G3, DMBA-induction only.*

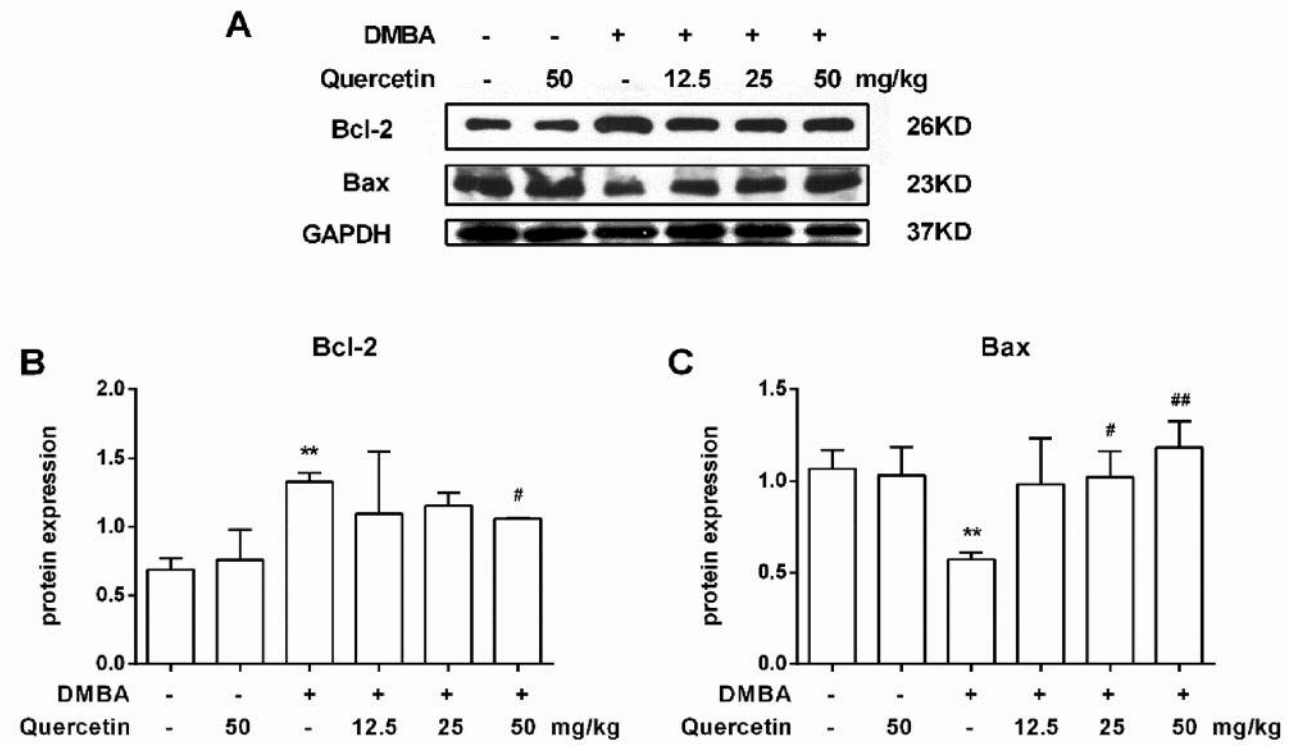

Figure 6. *Effect of quercetin on protein expression of Bcl-2 and Bax in DMBA-induced HBP carcinogenesis. A. Representative immunoblots of Bcl-2 and Bax proteins in each group. B. Bcl-2 protein expression in each group. C. Bax protein expression in each group. **p<0.01, when compared to the G1 normal-control group. #p<0.05 and ##p<0.01, compared to G3, DMBA induction only.*

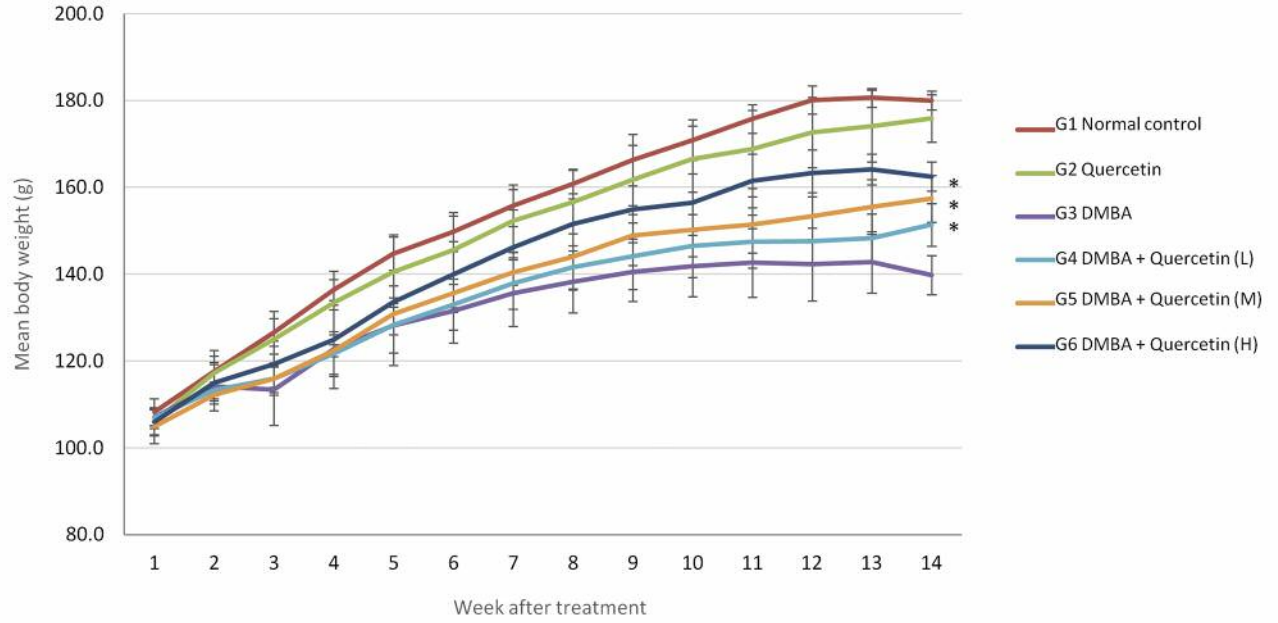

Figure 7. *Effect of quercetin on hamster body weight. Simultaneous treatment with quercetin significantly alleviated body weight loss in DMBA+quercetin groups. *p<0.05, compared to G3 DMBA induction only.*

Effect of quercetin on the body weight of the animals. As shown in Figure 7, final body weights were significantly decreased in all DMBA-treated animals (Group 3-6) compared to the normal control group ($p<0.01$). However, simultaneous treatment with quercetin significantly alleviated body-weight loss in DMBA+quercetin groups as compared to the DMBA-treated-only group (Group 3) ($p<0.05$). Quercetin treatment in normal animals (Group 2) did not affect animal body weight as compared to the normal control group ($p>0.05$).

In the present study, we reported for the first time that quercetin reduces tumor incidence and induces tumor apoptosis through the modulation of NF-κB signaling and its target genes in the DMBA-induced OSCC model.

Recent studies have indicated that NF-κB is strongly involved in the OSCC pathology (19). Our results showed that mRNA and protein expression of NF-κB p50 and p65 were increased in DMBA-induced HBP carcigenesis, further suggesting association of NF-κB activation with OSCC development. Simultaneous treatment with quercetin in DMBA-induced animals resulted in significant reduction of the increased expression of NF-κB p50 and p65, suggesting that suppression of NF-κB signaling is part of the anti-cancer mechanism of quercetin.

Our results showed that abrogation of the constitutive activation of NF-κB by quercetin was in turn associated with modulation in the transactivation of the NF-κB target genes Bcl-2 and Bax involved in apoptosis. In the present study, quercetin was found to induce apoptosis in DMBA induced cancer cells by upregulating the expression of Bax and reducing the expression of Bcl-2, suggesting that quercetin-induced suppression of NF-κB blocks the transactivation of its target genes implicated in oncogenesis.

In conclusion, quercetin reduces tumor incidence and induces cancer-cell apoptosis through modulation of NF-κB signaling and its target genes Bcl-2 and Bax in the DMBA-induced carcigenesis animal model, suggesting the potential of quercetin as a candidate for OSCC chemoprevention.

Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

Acknowledgements

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