Abstract. Background: Calcium glucarate (CGT) is a promising chemopreventive agent. This study evaluated the in vivo efficacy of CGT in preventing 7,12-dimethylbenz(α)anthracene (DMBA)-induced oral carcinogenesis in the hamster. Materials and Methods: Seventy-six Syrian hamsters were used, divided into four groups: group 1, untreated animals; 2, CGT controls; 3, DMBA-treated; 4, DMBA- and CGT-treated. Hamsters were painted three times weekly with 0.5% solution of DMBA and were fed a diet supplemented with CGT (64 mmol/kg, 2%). Animals were sacrificed at week 9 and 12 and pathology and histomorphometric analyses were performed. Results: At week 9, four dysplastic lesions and six carcinomas were identified in group 3 while only three dysplasias and five carcinomas were detected in group 4. At week 12, five animals of group 3 displayed a dysplasia, which was only detected in one animal of group 4. Squamous carcinomas were identified in all animals of both group 3 and 4. However, in group 3 four of the animals displayed multifocal lesions and carcinomas displayed histological features indicative of increased aggressiveness. Conclusion: The results obtained suggest that CGT can exert an inhibitory effect on oral carcinogenesis in the hamster and that further studies are warranted to evaluate its potential use as a chemopreventive agent in humans.

Incidence of oral squamous cell carcinomas, which frequently arise from leukoplakia, is increasing worldwide and its prognosis has not changed, unlike several other types of cancer, in the last few decades, since 50% of newly diagnosed patients die within a five-year period and the other 50% develop severe functional problems. Current therapies for leukoplakia, according to evidence-based medicine, have little influence on the natural history and progression of leukoplakia towards oral cancer, thus supporting the need for further studies to develop new chemopreventive and chemotherapeutic strategies (1-4).

Among the various natural compounds tested in experimental and clinical studies, calcium glucarate (CGT) has proved to be a promising chemopreventive agent for several tumor types including breast, colon, liver, lung and skin cancer. CGT is the calcium salt of D-glucaric acid, found in both animals and vegetables, and is also produced in small amounts in humans (5-8).

The hamster buccal pouch model is the most studied and best known animal model for oral squamous carcinogenesis since the tumors closely resemble human oral squamous cell tumors. For this reason, the hamster buccal pouch is an excellent model for morphological studies and for the evaluation of new potential chemopreventive agents. In this model, dysplasia develops after 6 to 8 weeks, tumors appear at 10 to 12 weeks and large invasive lesions appear at 12 to 14 weeks (9-12).

The aim of this study was to take advantage of the hamster buccal pouch model to evaluate the in vivo efficacy of CGT in preventing the onset and progression of experimentally induced oral carcinoma, as well as to study histomorphometric changes associated with the treatment.

Materials and Methods

Animals. Seventy-six three-month-old Syrian hamsters (Cricetus auratus), weight approximately 100 g were used, divided into four groups: group 1, untreated (n=16); 2, CGT alone (n=16); 3, DMBA-
treated (n=28); 4, DMBA- and CGT-treated (n=16) animals. Hamsters in groups 3 and 4 were painted three times weekly in their left buccal pouch with a 0.5% solution of 7,12-dimethylbenz(α)antracene (DMBA; Sigma-Aldrich Co., Saint Louis, MO, USA) in heavy mineral oil with a sable brush (No. 4). At each painting, about 1.2 mg of DMBA in oil was applied to the epithelial surface of the pouch (9, 10, 13). Simultaneously, the diet of animals in groups 2 and 4 was supplemented with 64 mmol/kg (2%) of CGT (Sigma-Aldrich Co., Saint Louis, MO, USA) (14). Hamsters were allowed to eat ad libitum and all groups were weighed every week (Cricetus auratus eats an average of 6 g/day on a laboratory diet thus ingesting an assumed dose of 0.12 g/day of CGT) (15). Two randomly selected hamsters from group 3 were sacrificed every week, starting from the fifth week, in order to follow more closely the epithelial changes associated with DMBA treatment and to identify the onset of pathological lesions. At week nine, dysplasias and tumors appeared and 50% of the animals from all groups were sacrificed. DMBA administration was discontinued at week nine (for both groups 3 and 4), whereas CGT administration continued until week twelve (for both groups 2 and 4), when the experiment was concluded.

**Histological and histomorphometric analysis.** The left buccal pouch mucosa was separated from the skin, photographed, fixed in formalin and embedded in paraffin. Seven sections were stained with periodic acid of Shiff (PAS) and with hematoxylin-eosin. Furthermore, each histological section of the pouch was histomorphometrically evaluated and analyzed using a Nikon Eclipse microscope, with a millimeter-graduated scale. Histological evaluation allowed the identification of epithelial changes as well as dysplastic and cancerous lesions which were histomorphometrically analyzed, according to parameters reported in Table I, in all animals sacrificed at week 9 and 12 (13, 16-18).

| Epithelium Dysplasia Carcinoma |
|-------------------------------|-----------------------------|-----------------------------|
| Thickness of the epithelium   | Thickness and width of the dysplastic area |
| Areas of hyperplasia and atrophy | Grade of dysplasia | Endophytic or exophytic feature of the tumor |
| Thickness of the keratin      | Diameter of the basal cell nuclei | Presence of infiltration |
| Thickness of SCT              | Thickness of SCT | Mean nuclear diameter |
| Diameter of the basal cell nuclei | Number of inflammatory cells infiltrating the underlying connective tissue | Thickness of the underlying connective tissue |
| Number of inflammatory cells infiltrating SCT | Volume of the tumor |

SCT: Subepithelial connective tissue.

Statistical analysis. Quantitative variables were tested for normal distribution. Non parametric variables were compared by means of Mann-Whitney test, for two independent samples, and by means of Kruskal-Wallis test, for several independent samples. Parametric variables were compared by means of two-tailed ANOVA: if this proved significant, a post hoc Bonferroni test was performed to define differences between groups. Significance at the p≤0.05 level was used to determine statistical significance. Statistical analysis was performed using the software Intercooled Stata 8.0 (Stata Corporation, College Station, TX, USA).

**Results**

**General remarks.** The scheme of the entire experiment is shown in Figure 1. Animals were weighed every week and no animals died prematurely. As shown in Figure 2, CGT treatment alone (group 2) had no effects on animal weight during the entire experiment, while DMBA treatment (group 3) was associated with a decrease of animal weight, which became evident after 4 weeks. This effect was slightly counteracted by CGT in group 4 receiving both DMBA and CGT. The first dysplastic and cancerous lesions were observed in group 3 after 9 weeks of treatment, while animals of groups 1 and 2 did not display any pathological change at weeks 9 nor at week 12.
Histopathology and histomorphometry at week 9. At week 9, in group 3 the mean epithelial thickness of the apparently normal, but DMBA-exposed epithelium and the SCT thickness appeared significantly increased, when compared with group 1 ($p<0.05$). In group 4, the mean epithelial thickness was also significantly increased ($p<0.05$), whereas SCT was similar to that of the control group 1 (Table II).

Slight changes observed in group 2 were not significant when compared to those of group 1. In both groups 3 and 4, areas of hyperplasia and atrophy were present. The thickness of the underlining SCT was significantly increased in group 3 animals, while it remained similar to the one underlining normal epithelium in the group 4 animals. Considering the underlining inflammatory response, the animals of group 4 presented the lowest infiltrate, even lower than that of the animals of group 3.

At week 9, four (4/8) animals of group 3 displayed a mild/moderate dysplasia which was also detected in three (3/8) animals of group 4. Neither group showed any severe dysplasia or carcinoma in situ. In group 4, the SCT underlining dysplastic areas were thinner and presented a lower inflammatory infiltrate than those in group 3 (Table III).

Squamous carcinomas were also identified in six animals of group 3 and five of group 4. When comparing histomorphometric features of tumors, all carcinomas of group 4 and 3 of group 3 were exophytic and the volume of tumors detected in group 4 was significantly smaller than that of those detected in group 3 (1.8 μm$^3$ vs. 34.1 μm$^3$; $p<0.03$) animals (Table IV and Figure 3).

Histopathology and histomorphometry at weeks 12. The discontinuation of DMBA from week 9 in group 3 was associated with a reduction in the SCT thickness (underlining all three types of epithelium: normal, hyperplastic and atrophic) towards the value of SCT in group 1 at week 12. On the other hand, the discontinuation of DMBA from week 9 with the uninterrupted administration of CGT in group 4 was associated with a marked increase of the SCT values (Table II).

Considering the inflammatory response, the animals of group 4 presented the lowest infiltrate, even lower than that of the animals of group 3, as already seen at week 9.

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**Table II. Histomorphometric data of normal-appearing epithelium (9th and 12th week). Values are the mean (±standard deviation).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>9th Week</th>
<th>12th Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (Control)</td>
<td>Group 2 (CGT)</td>
</tr>
<tr>
<td>Mean epithelial thickness (μm)$^a$</td>
<td>45.3 (3)</td>
<td>60.3 (13.6)</td>
</tr>
<tr>
<td>Hyperplastic epithelium (μm)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atrophic epithelium (μm)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCT thickness (μm) underlining normal epithelium$^b,e,h$</td>
<td>44.9 (4.9)</td>
<td>53.2 (10.3)</td>
</tr>
<tr>
<td>SCT thickness (μm) underlining hyperplastic epithelium$^d,g$</td>
<td>-</td>
<td>99.3 (46.2)</td>
</tr>
<tr>
<td>SCT thickness (μm) underlining atrophic epithelium$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Keratin thickness (μm)</td>
<td>13.7 (3.1)</td>
<td>16.5 (5.3)</td>
</tr>
<tr>
<td>Mean diameter of the basal nuclei (μm)</td>
<td>5.6 (0.4)</td>
<td>5.6 (0.4)</td>
</tr>
<tr>
<td>Inflammatory infiltrate$^c,f$</td>
<td>8.6 (2.5)</td>
<td>10.3 (2.8)</td>
</tr>
</tbody>
</table>

Each group consisted of eight animals; SCT: Subepithelial connective tissue; $^a$9th week: ANOVA $p<0.01$, Bonferroni: G1 vs. G3 $p<0.05$, G1 vs. G4 $p<0.05$; $^b$9th week: ANOVA $p<0.001$, Bonferroni: G1 vs. G3 $p<0.001$, G2 vs. G3 $p=0.002$, G3 vs. G4 $p<0.05$; $^c$9th week: ANOVA $p<0.001$, Bonferroni: G1 vs. G3 $p<0.001$, G2 vs. G3 $p=0.002$, G3 vs. G4 $p<0.05$; $^d$9th week: ANOVA $p<0.05$, Bonferroni: G1 vs. G3 $p<0.05$, G2 vs. G3 $p<0.001$; $^e$9th week: ANOVA $p<0.05$, Bonferroni: G1 vs. G3 $p<0.05$, G2 vs. G3 $p<0.001$; $^f$12th week: ANOVA $p<0.05$; Bonferroni: G1 vs. G3 $p<0.05$, G2 vs. G4 $p<0.001$; $^g$12th week $p<0.05$ (Mann-Whitney test); $^h$12th week $p<0.05$ (Kruskal-Wallis test).
Five (5/8) animals of group 3 displayed a mild/moderate dysplasia which was only detected in one (1/8) animal of group 4. Neither group showed any severe dysplasia or carcinoma in situ. In group 4, the SCT underlining dysplastic areas were thinner and presented a lower inflammatory infiltrate than those in group 3 (Table III). As already observed at week 9, the group 4 animals displayed a thinner SCT underlining dysplastic areas and a lower inflammatory infiltrate compared to those of group 3 (Table III).

Squamous carcinomas were identified in all animals of both group 3 and 4. However, four of the group 3 animals displayed multifocal lesions. All carcinomas of group 3 animals were exophytic, while three (3/12) of the malignant lesions detected in group 4 animals were endophytic (Table IV). Surprisingly, carcinomas of group 4 were larger than those of group 3 but this difference was not statistically significant.

**Overall histopathological and histomorphometrical evaluation.** Comparative analysis of histomorphometric features (diameter of nuclei and SCT inflammatory infiltration) between normal epithelium and cancer during the model of oral carcinogenesis utilized in this study, allows the following considerations. At week 9, larger nuclear diameters were observed in cancer cells of group 3 animals compared with those of normal epithelium (7.5 μm vs. 5.8 μm; \(p<0.05\)) and a more pronounced SCT inflammatory infiltrate was found under the cancer tissue compared to normal-appearing epithelium (22.3 vs. 6.6; \(p<0.003\)). When the same parameters were analyzed in group 4 animals, only SCT inflammatory infiltrate was significantly different being more pronounced in tumors compared to normal epithelium (21.6 vs. 4.2; \(p<0.05\)), while nuclear diameter showed no significant differences between epithelium and tumors (epithelium 6.1 μm vs. 6.8 μm) (Table IV).
Figure 3. Representative lesions detected after 9 weeks. a: Exophytic, well-differentiated carcinoma arising in group 3 (H&E, ×10). b: Detail of the invasive front of the carcinoma shown in (a); differentiated carcinoma cells with scattered mitoses, a diffuse lymphomonocytic inflammatory infiltrate and some large vessels can be seen (H&E, ×200). c: Exophytic, non-infiltrating carcinoma arising in group 4 with areas of dysplasia (H&E, ×10). d: At higher magnification, differentiated carcinoma cells are revealed (H&E, ×200).

Figure 4. Representative lesions detected after 12 weeks. a: Exophytic invasive carcinoma arising in group 3 (H&E, ×10). b: Detail of the invasive front of the carcinoma shown in (a); highly undifferentiated cells with atypical large nuclei, dyskaryosis and anisocytosis can be seen (H&E, ×200). c: Exophytic, papillary non-infiltrating carcinoma arising in group 4 (H&E, ×10). d: At higher magnification, differentiated carcinoma cells are revealed (H&E, ×200).
At week 12, only SCT inflammatory infiltrate displayed significant differences between epithelium and tumors in both group 3 (8.6 vs. 17.1; \( p<0.05 \)) and group 4 (4.4 vs. 16; \( p<0.05 \)), while no differences were detected in terms of nuclear diameter in either of the two groups (Table IV).

**Discussion**

Numerous *in vitro* and *in vivo* studies on CGT highlighted its potential as chemotherapeutic and chemopreventive agent, showing no side-effects. The purpose of our study was to evaluate the effect of CGT, using histological and histomorphometric criteria, in a hamster experimental model of oral carcinogenesis (5, 6, 8, 19).

The first important finding of this study was that simultaneous administration of CGT was associated with a reduced number of DMBA-induced tumors at both week 9 and 12 (Table IV), thus confirming a chemopreventive effect of CGT in this experimental model.

Histomorphometric analyses also showed that at week 9, the tumors which had developed in group 4 (treated with DMBA+CGT) were smaller than those detected in group 3 (treated with DMBA alone) animals (\( p<0.03 \)), with smaller nuclei and a thicker SCT (Table IV). At week 12, carcinomas in group 4 were all exophytic and some of them did not infiltrate the SCT, while group 3 animals developed more numerous and more aggressive carcinomas, some of them endophytic and all infiltrating the SCT (Table IV). A controversial finding was the presence, at 12 weeks, of larger carcinomas in group 4, compared to group 3, even though this difference was not statistically significant. However, it is noteworthy that tumors in group 4 animals never reached the volume observed in group 3 at week 9 while the reduction in tumors volume observed in group 3 between weeks 9 and 12 was likely related to the general compromised conditions of the animals in group 3, as previously mentioned (Figure 2).

Another interesting finding of this study is that group 4 animals also developed fewer dysplastic lesions than group 3 both at weeks 9 and 12. Dysplasias at week 9 in group 4 presented a significantly thinner SCT and a significantly lower inflammatory infiltrate than group 3. The detection of just one dysplastic lesion at week 12 in group 4, made the statistical analysis impossible, but confirmed the protective role of CGT.

Overall, our findings support the potential of CGT for preventing DMBA-induced oral carcinogenesis, reducing the number of dysplastic lesions, as well of tumors and their histological aggressiveness (less infiltration).

Nuclear size is a well known marker of the aggressiveness of cancer cells. We found that the nuclear diameter showed a statistically significant increase during the shift from normal epithelium to carcinoma in group 3 animals, whereas in group 4 animals, CGT was able to prevent these changes (with the nuclear diameter of tumors at week 9 being similar to those of the normal epithelium). On the other hand, GCT appeared not to be able to impair the inflammatory response against tumors since inflammatory infiltrate, which was higher under tumors compared to normal epithelium, was comparable in both group 3 and 4 animals (Table IV) (7, 20, 21).

DMBA-exposed epithelium (apparently normal) displayed some alterations following DMBA treatment: *e.g.* increased epithelial thickness and SCT thickness (Table II). At week 9, the simultaneous treatment with CGT did not prevent the increase in the total thickness of the epithelium, whereas it appeared to exert an important effect on the SCT thickness which was not different between the group 4 and the group 1 control animals (under normal, hyperplastic and atrophic epithelium). We consider more reliable the findings of week 9, since those of week 12 might have been biased by the general compromised conditions of the animals in group 3, as previously mentioned (Figure 2).

**Discussion**

Profound immunosuppression following treatment with DMBA has been previously documented. It is of interest that CGT did not impair the inflammatory response against tumors (Table IV), but it remains to be defined whether CGT chemopreventive action might also involve the immune system (22, 23).

When considering merely clinical parameters, such as the weight of the animals (Figure 1), CGT at the dose used in this study not only did not display a toxic effect (the animals of group 2 gained weight in the same way as those of group 1) but it also exerted a mild protective effect against DMBA-induced weight loss (compare group 4 vs. group 3). These data are in line with those available in the literature. In this regard, we used a 64 mmol/kg CGT diet, which some authors consider sub-optimal (usually a 128 mmol/kg diet is used for chemoprevention), but it must be considered that the majority of these studies used the Sprague-Dawley rat as an experimental model, which is a more resistant animal than *C. auratus* (13, 14, 23).

Considering the CGT pharmacodynamic, its effect is exerted through two main mechanisms: *i)* inhibiting cellular β-glucuronidase and *ii)* inhibiting β-glucuronidase of the colonic microflora. This enzyme deconjugates the glucuronate compounds, diminishing the cellular excretion on one hand, and favoring a enterohepatic circulation on the other. It is thus possible that CGT could exert a double effect in the hamster buccal pouch model, favoring the excretion of DMBA from the oral epithelial cells which are directly in contact with DMBA and inhibiting the enterohepatic circulation, thus favoring the excretion of the DMBA portion which is ingested by the hamsters after the buccal pouch painting (5, 6, 24).

In conclusion, regardless of the underlying molecular mechanisms, the results of the present study demonstrate that CGT is able to inhibit carcinogenesis in the hamster buccal...
pouch model without significant toxic effects. These findings confirm the reported chemopreventive and chemotherapeutic activity of CGT and show that further studies to evaluate its usefulness in humans are warranted.

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**References**


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