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Placental glutathione S-transferase isoenzyme expression in polycyclic aromatic hydrocarbon-induced hamster buccal pouch mucosa

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Abstract

Placental glutathione S-transferase (GST-P) immunohistochemical activity in polycyclic aromatic hydrocarbon (PAH)-induced hamster buccal pouch mucosa (HBPM) was investigated. Equimolar concentrations of 3,4-benzo[a]pyrene (BP), benz[a]anthracene (BA) and 20-methylcholanthrene (MC) in mineral oil solution were applied three times per week for up to 20 weeks to bilateral pouches of the hamsters. Control pouches were treated with 7,12-dimethylbenz[a]anthracene (DMBA), turpentine and mineral oil, respectively. A significant increase in the number of GST-P foci in the dysplastic pouches was found when compared with the hyperkeratotic and hyperplastic pouches after 5 weeks' DMBA treatment. The average numbers of GST-P positive stainings were significantly higher in DMBA-treated groups than in BP-, BA- and MC-treated groups killed at 5, 20, 30 and 40 weeks. No GST-P positivity was found in any pouches treated with turpentine and mineral oil observed at various periods. These results highlight the carcinogen-specificity of GST-P chemically induced in HBPM and indicate that induction of GST-P in pouch mucosa is linked with the carcinogenic potency of PAHs. The present study underpins the hypothesis that GST-P chemically induced in HBPM is an early marker of ongoing squamous cell carcinogenesis.

Keywords: GST-P; Polycyclic aromatic hydrocarbons; Hamster

1. Introduction

Glutathione S-transferases (GSTs) of rat and human are the products of a multigene family. Rat GSTs comprise at least 12 distinguishable classes of isoenzymes, the most extensively characterised of which is the placental class (GST-P) [1]. Cytosolic human GSTs, divided into four classes, called α, μ, π [2] and θ [3], have been identified in various organs. Human GST π is related to rat GST-P in enzymatic and immunologic properties. A well-established function of GSTs is their carcinogen-metabolising ability by catalysing the conjugation of potentially mutagenic electrophilic substrates with glutathione, thereby enhancing the elimination of the potentially deleterious compounds as water soluble molecules [4].

Polycyclic aromatic hydrocarbons (PAHs) are well-known complete chemical carcinogens capable of tumour initiation, promotion and progression [5]. PAHs are metabolised to reactive intermediates that bind to DNA to exert their carcinogenic actions [6].

The hamster buccal pouch mucosa (HBPM) is one of the most widely accepted experimental models of oral carcinogenesis [7] which closely resembles human lesions [8]. Previous studies [9,10] have documented enhanced levels of GST-P in HBPM treated with 7,12-dimethylbenz[a]anthracene (DMBA), which belongs to the group of PAHs. However, it is speculated that GST-P expression may be associated with the strong toxic effect of DMBA and not be carcinogenic specific. Furthermore, to the best of our knowledge, the induction of GST-P in PAH-treated pouches, other than DMBA, has not been studied. Therefore, in the current study, GST-P expression in HBPM treated with equimolar concentrations of three much less toxic carcinogenic PAHs (benz[a]anthracene (BA), 1,4-benzopyrene (BP), 20-methylcholanthrene (MC)) [11] was investigated;using DMBA as the positive control. The possibilities of GST-P expression in HBPM upon treatment with turpentine [12], a non-carcinogenic inflammatory

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hyperplasiogenic agent, and the application of mineral oil, a non-toxic chemical, were also evaluated. The aim was to examine the hypothesis that GST-P chemically induced in HBPM is an early marker of ongoing squamous cell carcinogenesis. If our hypothesis is correct, induction of GST-P in this animal model should be carcinogen-specific and the frequency of GST-P expression in PAH-exposed HBPM corresponds with the carcinogenic potency of PAHs.

2. Materials and methods

2.1. Hamsters and treatments

BP, BA, MC and DMBA procured from Sigma Chemical Company (St. Louis, Missouri, U.S.A.), as well as turpentine, purchased from Stevens Chemical Company (Christchurch, New Zealand), were of the highest purity commercially available. The protocol ensured humane practices. Two hundred and forty non-inbred young (6 weeks old) male Syrian golden hamsters (purchased from National Science Council Animal Breeding Centre, Republic of China), weighing approximately 100 g at the commencement of the experiment, were randomly divided into six groups, each containing 40 animals. The animals were maintained under constant conditions (22°C, 12:12 h light/dark cycle) in an air-conditioned animal house. They were fed with standard laboratory pellets and tap water *ad libitum*. After allowing 1 week to acclimatise to the new surroundings, equimolar concentrations (20 mM in mineral oil) of BP (40 hamsters), BA (40 hamsters), MC (40 hamsters) and DMBA (40 hamsters) solutions were painted three times per week (09.00 h on Monday, Wednesday and Friday) to the medial walls of both buccal pouches with six strokes of a no. 4 sable-hair brush which had been wiped against the side of the container containing the solution [8]. Bilateral pouches of a group of 40 animals were treated three times per week with turpentine (50% v/v in mineral oil), whereas another group of 40 animals was similarly treated with mineral oil.

At the end of 5 weeks (3 days following the last treatment), 10 animals from each group were withdrawn randomly from the painting and euthanised by a lethal dose of diethyl ether at the same time (09.00 h) of the day to avoid the influence of diurnal variation. The killed hamsters were fixed in a surpine position with pins. The bilateral pouches were exposed by dissection, cut from their oral openings to their caudal ends along the middle of their lateral walls and examined grossly. Subsequently, both pouches were excised and placed on card papers to prevent distortion of the specimens.

The application of the chemicals to the remaining hamsters in each group continued until 20 weeks. At 20 weeks, treatment with the chemicals was terminated and 10 animals from each group were killed with procedures identical to those described above. The remaining 20 animals in each group were maintained without further treatment, 10 of which from each group were subsequently killed at 30 weeks. The last 10 animals from each group were finally killed at 40 weeks.

Four specimens of approximately 1 cm in length were taken from each pouch. They were routinely processed for light microscopy. They were fixed in 10% neutral buffered formalin solution, dehydrated in ascending alcohols, cleared in xylene and embedded in paraffin. Serial sections (4 μm thick) of each specimen were then cut. One section was prepared for GST-P immunohistochemistry, while another was used for haematoxylin–eosin staining. Therefore, four slides from each pouch and eight slides from each animal were used for GST-P immunostaining.

2.2. Immunohistochemical staining of GST-P

Immunohistochemical staining of GST-P isoenzyme was performed, as previously described [10], by the avidin–biotin–peroxidase complex (ABC) technique according to Hsu et al. [13]. Polyclonal antibodies of rat GST-P were raised on rabbits [14] and obtained from Biotrin International Laboratory (Dublin, Ireland; Cat. no. BIO23 Yp(r)). The antisera do not cross-react with GST α or GST μ isoenzymes but show strong cross-reaction with human and mouse π isoenzymes (information from manufacturer). The total number of GST-P positive foci for each animal was the sum of the number of stained foci from the eight representative slides from both treated pouches. Negative controls were included by substituting the antisera with pre-immune rabbit sera. The significance of difference between the average numbers of GST-P stained foci in the various groups was assessed using the two-tailed Student's *t*-test for paired data.

3. Results

3.1. Gross observations

Pouches treated with BP, BA and MC were grossly flat and tumour free after 5 and 20 weeks of treatment. Observation without further treatment up to 40 weeks showed that pouches treated with BP, BA and MC for 20 weeks were still grossly free of tumour formation. A thickened mucosa with a rough surface and whitish granular appearance was observed in the pouches treated with DMBA for 5 weeks. Extensive tumour formation with a 100% tumour incidence developed in the pouches treated with DMBA for 20 weeks. Weight loss, malnutrition and sometimes cachexia were noted in

many DMBA-treated animals at the end of 20 weeks of exposure to the carcinogen when compared with the other experimental groups. Gross examinations of all pouches treated with mineral oil and turpentine revealed no obvious changes at various periods.

3.2. Histopathology

No carcinomas, but occasionally dysplasias, were noted in the histological sections of the pouch tissues of hamsters treated with BP, BA and MC for 5 and 20 weeks, as well as in hamsters treated with BP, BA and MC for 20 weeks and then maintained without further treatment, and killed at 30 and 40 weeks. Hyperkeratoic, hyperplastic and dysplastic pouches were seen after 5 weeks' DMBA treatment. After 20 weeks' DMBA application, invasion of tumour cells into the underlying connective tissue was observed. No apparent histological changes were observed in all the pouches treated with mineral oil for 5 and 20 weeks, as well as in the pouches treated with mineral oil for 20 weeks and then observed without further treatment for up to 30 and 40 weeks. Epithelial hyperplasia and hyperkeratosis were noted in the pouches treated with turpentine for 5 and 20 weeks, but regression was noticed in the pouches treated with turpentine for 20 weeks and then without further treatment and subsequently killed at 30 and 40 weeks.

3.3. GST-P immunohistochemistry

The possible patterns of GST-P positivity in the PAH-treated pouch mucosa area are shown in Fig. 1. A positive focus may vary from a single cell to a cluster of a various number of cells without the interruption of unstained cells.

GST-P positivity of BP-, BA- and MC-treated pouches (killed at 5, 20, 30 and 40 weeks) was most likely to be multiple single positive cells, located randomly in the mucosa (Fig. 2a–c). A cluster of positive-stained cells was occasionally observed near the basal layer (Fig. 2a–c). Both cytoplasmic (Fig. 2b) and nuclear staining (Fig. 2a–c) were observed. The average numbers of GST-P stained foci were significantly higher in the BP-, BA- and MC-treated groups killed at 20, 30 and 40 weeks than those observed at 5 weeks [Table 1(∗); $P < 0.05$, Student's t-test]. The mean numbers of GST-P stained foci did not vary significantly in BP-, BA- and MC-treated hamsters killed at 20, 30 and 40 weeks [Table 1(—∗∗—); $P > 0.05$, Student's t-test]. The average numbers of GST-P positive staining among the hamsters treated with BP, BA and MC for 5 weeks were similar to each other (Table 1, □). Additionally, all the mean numbers of GST-P stained foci within the BP-, BA- and MC-treated hamsters killed at 20, 30 and 40 weeks were close to each other (Table 1, □).

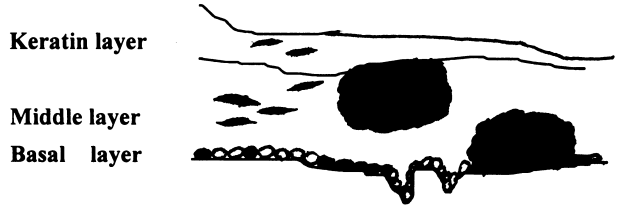

Fig. 1. A line drawing to illustrate the various patterns of placental glutathione S-transferase (GST-P) stained foci in the polycyclic aromatic hydrocarbon (PAH)-treated pouch mucosa (black shaded areas represent GST-P positivity). A positive focus, randomly located in a pouch mucosa, may vary from a single cell to a cluster of a various number of cells without the interruption of unstained cells.

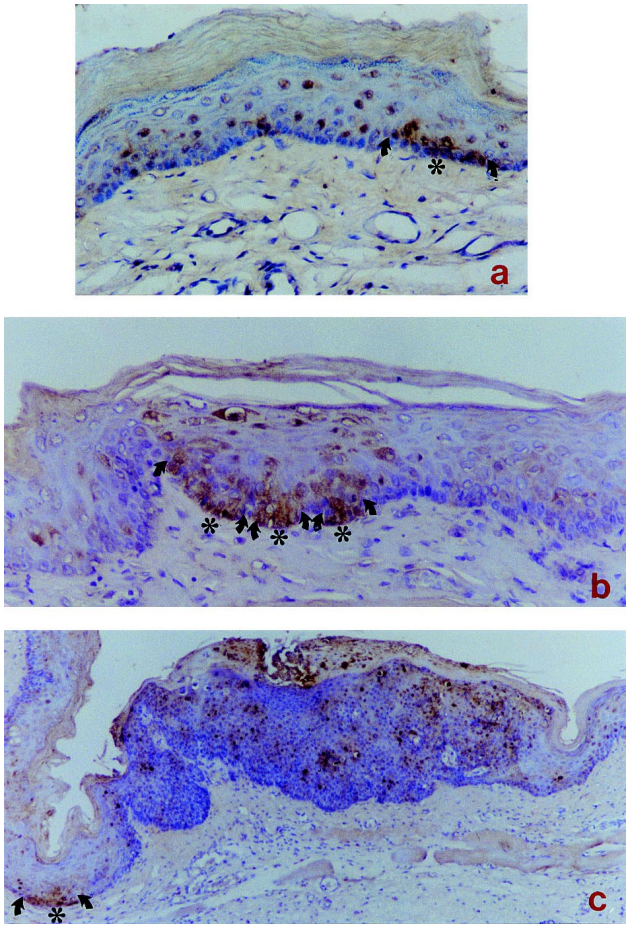

Fig. 2. (a) Placental glutathione S-transferase (GST-P) positivity in a pouch treated with 3,4-benzo[a]pyrene (BP) for 20 weeks. The foci chiefly comprised multiple single positive cells located randomly in the mucosa. A small cluster of several cells could also been seen near the basal layer (↗∗↖) (avidin–biotin–perioxidase complex (ABC) stain, ×100). (b) GST-P positivity in a pouch treated with benz[a]anthracene (BA) for 20 weeks. Three adjacent foci were observed near the basal layer (↗∗↖). Both cytoplasmic and nuclear stainings could be observed (ABC stain, ×100). (c) GST-P positivity in a dysplastic pouch treated with 20-methylcholanthrene (MC) for 20 weeks. The foci comprised multiple nuclear staining cells distributed randomly within the pouch mucosa. A small focus could also been seen near the basal layer (↗∗↖) (ABC stain, ×40).

Both cytoplasmic and nuclear staining were observed in the DMBA-treated pouches (Fig. 3a–d) with the patterns of positive foci similar to previous findings [9,10]. GST-P positivity was induced in the hyperkeratotic (Fig. 3a), hyperplastic (Fig. 3b) and dysplastic (Fig. 3c) pouches after 5 weeks' DMBA treatment. There was a significant increase in the number of GST-P foci in the dysplastic pouches when compared, respectively, with the hyperkeratotic and hyperplastic pouches after 5 weeks' DMBA application (Table 2). Additionally, there were significant increases in the average number of GST-P positive stainings in DMBA-treated hamsters killed at 20, 30 and 40 weeks as compared with 5 weeks' DMBA treatment [Table 1(*); $P < 0.05$, Student's t-test]. No significant alterations in the average numbers of

Table 1
The average numbers of placental glutathione S-transferase (GST-P) stained foci per hamster in 7,12-dimethylbenz[a]anthracene (DMBA), 3,4 benzo[a]pyrene (BP), benz[a]antharacene (BA) and 20-methylcholanthrene (MC)-treated groups

Chemicals	Killing periods: 5 weeks	20 weeks	30 weeks	40 weeks
DMBA	122±77[a]	270±86 —**—	286±75 —**—	292±96
BP #	32±12	87±23 —**—	92±37 —**—	84±36
BA #	28±14	75±24 —**—	88±36 —**—	94±39
MC #	36±29	90±31 —**—	97±47 —**—	98±44

* Statistical significance ($P < 0.05$, Student's t-test) existed in the average numbers of GST-P positive foci in the DMBA-treated hamsters killed at 20, 30 and 40 weeks when compared with hamsters killed at 5 weeks; note also that the average numbers of GST-P positive foci in the non-DMBA-treated groups observed at 20, 30 and 40 weeks were significantly higher than those observed at 5 weeks ($P < 0.05$, Student's t-test).

Statistical increase existed when the average numbers of GST-P positive foci in the DMBA-treated hamsters were compared with the non-DMBA (BP, BA and MC)-treated hamsters killed at 5 weeks ($P < 0.05$, Student's t-test). Note also that statistical increases exist when the average numbers of GST-P positive foci in the DMBA-treated hamsters were compared with the non-DMBA-treated hamsters killed at 20, 30 and 40 weeks ($P < 0.05$, Student's t-test).

—**—: statistically insignificant ($P > 0.05$, Student's t-test). Values of means within boxes were similar to each other.

[a] Mean ± standard deviation.

Table 2
The average numbers of placental glutathione S-transferase (GST-P)-stained foci per hamster in hyperkeratotic, hyperplastic and dysplastic pouches after 5 weeks of 7,12-dimenthylbenz[a]anthracene (DMBA) treatment

Pouches	Average no. of GST-P foci
Hyperkeratotic	22 ± 9*[a]
Hyperplastic	34 ± 10
Dysplastic	66 ± 14*

* Statistical increase was noted when compared with hyperkeratotic hyperplastic pouches ($P < 0.05$, Student's t-test).

[a] Mean ± standard deviation.

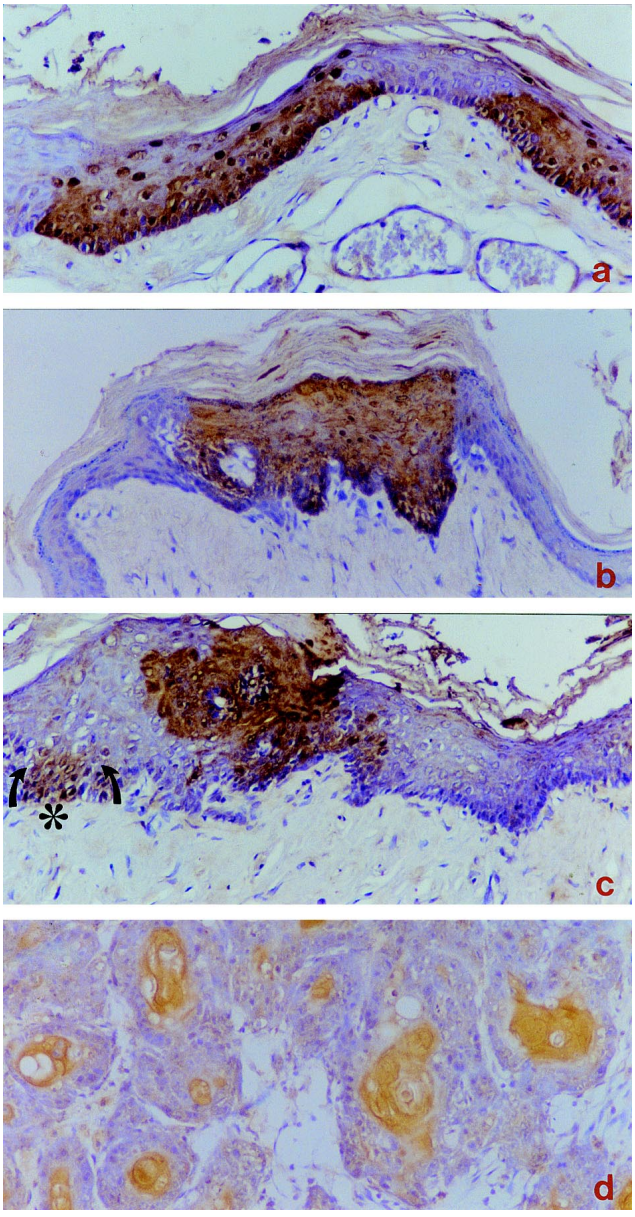

Fig. 3. (a) Placental glutathione S-transferase (GST-P) positivity in a hyperkeratotic pouch treated with 7,12-dimethylbenz[a]anthracene DMBA for 5 weeks. Two foci involved the lower portion of the pouch mucosa could be seen. Multiple single cells with nuclear staining were also observed in the upper portion of the mucosa avidin–biotin–peroxidase complex (ABC stain, ×100). (b) GST-P positivity in a hyperplastic pouch treated with DMBA for 5 weeks. There was a positive focus involved the whole layer but spared the keratin layer, which contained a few positive cells (ABC stain, ×100). (c) GST-P positivity in a dysplastic pouch treated with DMBA for 5 weeks. A large focus involved the upper portion of the mucosa could be seen. A smaller, weaker stained, focus was also observed near the basal layer (↗*↖) (ABC stain, ×100). (d) GST-P staining in an invasive squamous cell carcinoma in a pouch treated with DMBA for 20 weeks (ABC stain, ×100).

GST-P stained foci in DMBA-treated hamsters were observed at 20, 30 and 40 weeks [Table 1(—**—); $P > 0.05$, Student's t-test], whereas statistically significant increases were noted when the mean numbers of GST-P positive foci in the DMBA-treated groups were compared with the BP-, BA- and MC-treated groups observed at 5, 20, 30 and 40 weeks [Table 1(#); $P < 0.05$, Student's t-test]. No GST-P positivity was seen in any pouches treated with turpentine and mineral oil observed at 5, 20, 30 and 40 weeks. The control sections, where the antisera were replaced with preimmune rabbit sera, demonstrated negative findings.

4. Discussion

Enhanced levels of GST-P in DMBA-treated HBPM have been shown in previous studies [9,10]. However, the elevated expression may be associated with the strong toxicity of DMBA. The present study is a very important extension of the previous work [9,10] as it further supports and clarifies the suggestion that the induction of GST-P in HBPM is carcinogenic specific; underpinning the hypothesis that GST-P chemically induced in HBPM is an early marker of ongoing squamous cell carcinogenesis. Additionally, these data are also compatible with the results of our previous studies [15,16] of placental GST on human oral (pre)malignant lesions.

To the best of our knowledge, the induction of GST-P by PAHs, besides DMBA, has not been previously demonstrated in HBPM. Of the four PAHs evaluated in the present study, DMBA induced a considerable quantity of GST-P foci in HBPM as compared with BP-, BA- and MC-treated pouch mucosa. Therefore, the current results not only demonstrate that GST-P is induced by PAHs other than DMBA in HBPM, but also indicate that the frequency of GST-P induced in PAH-exposed HBPM corresponds with the carcinogenic potency of PAHs.

Our results revealed that the induction of GST-P stained foci after 20 weeks' treatment was independent of the painting period: the average numbers of GST-P stained foci already approached plateau values after 20 weeks' DMBA treatment as well as BP, BA and MC treatments. This may be attributed to the "saturation effect" that almost all the keratinocytes in HBPM have already responded to the carcinogenicity of DMBA, BP, BA and MC [10].

The induction of GST-P by PAHs, as shown in the present study, could increase the ability of pouch keratinocytes to resist the burden of PAHs; reducing the risk of carcinoma development. However, the role of GST-P in models of chemical carcinogenesis may be its permissive effect on cell cycle activity and downregulation of apoptosis; allowing proliferation of the initiated cells [17]. Therefore, GST-P may alternatively confer an anticarcinogenic defence mechanism on the normal pouch keratinocytes on exposure to PAHs and provide a protection against the carcinogenic potency of PAHs. However, the same isoenzyme, GST-P, induced in HBPM, may at least in part, render the initiated pouch keratinocytes more vulnerable to the clonal expansion [17].

It is worth noting that GST-P was expressed in the lesions of hyperkeratosis and epithelial hyperplasia after 5 weeks' DMBA treatment but did not appear in the histologically similar lesions resulting from treatment with turpentine. This intriguing feature may further highlight the carcinogen-specificity of GST-P chemically induced in HBPM. The exact mechanism of the action of turpentine is still obscure. However, it has been described as a chemical irritant [18]. Our results confirmed that turpentine would be an appropriate hyperplasiogenic agent in HBPM; consistent with a number of previous studies [13,19,20] and also compatible with the cutaneous hyperplasia induced in the turpentine-treated mouse skin [18].

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References

[1] Sato K. Glutathione transferases as markers of preneoplasia and neoplasia. Advances in Cancer Research 1989;52:205–255.
[2] Mannervik B, Alin P, Gutherberg C, et al. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proceedings of the National Academy of Science USA 1985;82:7202–7226.
[3] Meyer DH, Coles B, Pemble SE, et al. Theta, a new class of GSH transferases purified from rat and man. Biochemical Journal 1991;274:409–414.
[4] Mannervik B. The isoenzymes of glutathione transferase. Advances in Enzymology Related Areas and Molecular Biology 1985;57:357–417.
[5] Pitot HC, Dragan YP. Chemical carcinogenesis. In: Klassen CD, editor. Casarett and Doull's Toxicology. New York: McGraw-Hill, 1996. p. 201–267.
[6] Thakker DR, Yagi H, Levin W, et al. Polycyclic aromatic carbons: metabolic activation to ultimate carcinogens. Bioactive Foreign Compounds 1985;7:177–242.
[7] Gimenez-Conti IB, Slaga TJ. The hamster cheek pouch carcinogenesis model. Journal of Cellular Biochemistry 1993;17F (Suppl.):83–90.
[8] Morris AL. Factors influencing experimental carcinogenesis in the hamster cheek pouch. Journal of Dental Research 1961;40:3–15.
[9] Zhang L. The value of placental glutathione S-transferase and γ-glutamyl transpeptidase as markers of altered foci during hamster pouch carcinogenesis. Carcinogenesis 1994;15:105–109.

[10] Chen YK, Lin LM. Sequential expression of placental glutathione S-transferase (GST-P) during DMBA-induced hamster buccal pouch squamous cell carcinogenesis. Journal of Oral Pathology and Medicine 1996;25:388–394.
[11] Solt DB, Polverinil PJ, Calderon L. Carcinogenic response of hamster buccal pouch epithelium to 4 polycyclic aromatic hydrocarbons. Journal of Oral Pathology 1987;16:294–302.
[12] Craig GT, Franklin CD. The effect of turpentine on hamster cheek pouch mucosa: a model of epithelial hyperplasia and hyperkeratosis. Journal of Oral Pathology 1977;6:268–277.
[13] Hsu SM, Raine L, Fanger H. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. Journal of Histochemistry and Cytochemistry 1981;29:577–580.
[14] Rushmore TH, Harris L, Nagai M, et al. Purification and characterization of P-52 (glutathione S-transferase-P or 7-7) from normal liver and putative preneoplastic liver nodules. Cancer Research 1988;48:2805–2812.
[15] Chen YK, Lin LM. Immunohistochemical demonstration of epithelial glutathione S-transferase isoenzymes in normal, benign, premalignant and malignant human oral mucosa. Journal of Oral Pathology and Medicine 1995;24:316–321.
[16] Chen YK, Lin LM. Evaluation of glutathione S-transferase activity in human buccal epithelial dysplasias and squamous cell carcinomas. International Journal of Oral and Maxillofacial Surgery 1997;27:205–209.
[17] Macaughan FM, Brown AL, Harrison DJ. The effect of inhibition of glutathione S-transferase P on the growth of the Jurkat human T cell line. Journal of Pathology 1994;172:357–362.
[18] Tarin D. Further electron microscopic studies on the mechanism of carcinogenesis: the specificity of the changes in carcinogen-treated mouse skin. International Journal of Cancer 1968;3:734–742.
[19] Shearer BH, McMillian MD, Jenkinson HF. Comparison of the effects of four hyperplastic agents on hamster cheek pouch mucosa. Journal de Biologie Buccale 1991;19:315–318.
[20] Shearer BH, Jenkinson F, McMillian MD. Changes in cytokeratins following treatment of hamster cheek pouch epithelia with hyperplastic or neoplastic agents. Journal of Oral Pathology and Medicine 1994;23:149–155.