available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Development of a model of tissue with potentially malignant disorders (PMD) in the hamster cheek pouch to explore the *long-term* potential therapeutic and/or toxic effects of different therapeutic modalities

Elisa M. Heber [a], *Andrea Monti Hughes* [a], *Emiliano C.C. Pozzi* [a,b], *Maria E. Itoiz* [a,c], *Romina F. Aromando* [c], *Ana J. Molinari* [a], *Marcela A. Garabalino* [a], *David W. Nigg* [d], *Verónica A. Trivillin* [a], *Amanda E. Schwint* [a,*]

[a] *Department of Radiobiology, National Atomic Energy Commission, Av. General Paz 1499, B1650KNA San Martin, Provincia de Buenos Aires, Argentina*

[b] *Research and Production Reactors, National Atomic Energy Commission, Argentina*

[c] *Department of Oral Pathology, Faculty of Dentistry, University of Buenos Aires, M.T. de Alvear 2142, 1122 Buenos Aires, Argentina*

[d] *Idaho National Laboratory, 2525 North Fremont Street, P.O. Box 1625, Idaho Falls, ID 83415, USA*

ARTICLE INFO

Article history:
Accepted 26 October 2009

Keywords:
Potentially malignant disorders
PMD
Hamster cheek pouch
Carcinogenesis protocols
Long-term follow-up

ABSTRACT

Objective: Given that locoregional recurrences developing from a tissue with potentially malignant disorders (PMD) in oral mucosa are a frequent cause of therapeutic failure, and that tissue with PMD is dose-limiting, the aim of the present study was to develop a model of tissue with PMD to evaluate the *long-term* therapeutic/toxic effects of different therapeutic modalities.

Materials and methods: We evaluated 5 carcinogenesis protocols based on topical application of the carcinogen dimethyl-1,2-benzanthracene in the hamster cheek pouch, twice a week for 4, 6, 7, and 8 weeks and the classical 3 times a week for 12 weeks.

Results: *Long-term* follow-up (8 months after protocol completion) was only possible with the 4- and 6-week carcinogenesis protocols. Tumour development increased progressively with time and aggressiveness of the carcinogenesis protocols. The time at which tumours developed in ≥90% of the animals was at protocol completion (T0) for the 12-week protocol, 1 month post-T0 for the 8-week protocol, 3 months post-T0 for the 7-week protocol and 4 months post-T0 for the 6-week protocol. <40% of the animals in the 4-week protocol developed tumours within the 8 months follow-up period. DNA synthesis rose as a function of time and protocol aggressiveness.

Conclusions: The 6-week carcinogenesis protocol was selected for *long-term* studies of different therapeutic modalities in tissue with PMD because it permitted *long-term* follow-up and guaranteed tumour development in ≥90% of the animals.

* *Corresponding author*. Tel.: +54 11 6772 7149; fax: +54 11 6772 7188.
E-mail address: schwint@cnea.gov.ar (A.E. Schwint).

0003–9969/$ – see front matter
doi:10.1016/j.archoralbio.2009.10.010

1. Introduction

The search for new therapeutic strategies for head and neck Squamous Cell Carcinoma (SCC) is warranted in view of the relatively poor 5-year survival rate of 52% and the large tissue defect caused by radical surgery.[1] The Syrian golden hamster cheek pouch oral carcinogenesis model is the best known animal system that closely mimics events involved in the development of premalignant and malignant human oral lesions.[2–4] Despite the remarkable success of Boron Neutron Capture Therapy (BNCT) protocols employed in studies by our group to treat the hamster cheek pouch tumours (65–93% tumour control with no normal tissue radiotoxicity),[5–8] a still unresolved challenge lies in controlling tissue with potentially malignant disorders (PMD).[9,10] Second primary tumour locoregional recurrences that arise in field-cancerized tissue are a frequent cause of therapeutic failure.[11] Furthermore, oral tissue with PMD (termed precancerous tissue in previous studies) behaves as a dose-limiting tissue in our experimental BNCT tumour control studies (e.g. [7]). Thus, therapeutic strategies aimed at controlling tumour or tissue with PMD must not exceed the tolerance of tissue with PMD. Within this context, the hamster cheek pouch oral cancer model poses a unique advantage in that tumours are induced by periodic, topical application of the carcinogen dimethyl-1,2-benzanthracene (DMBA), a process that mimics the spontaneous process of malignant transformation. Carcinogenesis protocols lead to the development of what has been termed herein tissue with PMD in keeping with Warnakulasuriya et al.[9] and Napier and Speight,[10] which in turn gives rise to the formation of tumours. Thus, this mode of tumour induction provides a model of tumour surrounded by tissue with PMD,[12,13] allowing for the study of therapeutic and/or toxic effects in tissue with PMD. Translational studies have been carried out by other groups employing a variety of experimental tumour models that do not give rise to the development of tissue with PMD because they are based on the implantation of tumour cells in normal tissue (e.g. [14]).

Our tumour control studies[5–7] were performed employing the classical carcinogenesis protocol that involves topical application of DMBA in the hamster cheek pouch 3 times a week for 12 weeks. Employing this standard carcinogenesis protocol that we routinely employ for *short-term* tumour control studies, we also analyzed the *short-term* effect of BNCT on tissue with PMD around tumours.[15] Although the results were encouraging, the marked aggressiveness of this carcinogenesis protocol yields an oral cancer model that is adequate for our *short-term* tumour control studies but is inadequate for *long-term* studies of tissue with PMD.

The aim of the present study was to develop a model of tissue with PMD that would serve to evaluate the *long-term* therapeutic/toxic effect of BNCT or other therapeutic modalities.

2. Materials and methods

We evaluated 5 carcinogenesis protocols based on the topical application of 5% of the carcinogen dimethyl-1,2-benzanthracene (DMBA) in mineral oil in the right cheek pouch of Syrian hamsters twice a week for 4, 6, 7, and 8 weeks or 3 times a week for 12 weeks. The 12-week protocol is the ''classical'' protocol we use for our *short-term* BNCT tumour control studies. Local and institutional guidelines were followed throughout to ensure the ethics of the research and the welfare of the animals.

Potential tumour development from tissue with PMD was assessed weekly by visual inspection and tumour volume assay (when pertinent) for 1–8 months after protocol completion, according to the clinical feasibility of follow-up for each group. The clinical signs and body weight of the animals were monitored regularly.

Tumour development from tissue with PMD was assessed at T0 (1 week after completion of the carcinogenesis protocol) and thereafter in terms of: percentage of animals that developed tumours at different time-points; T50 (the time to the development of tumours in 50% of the hamsters) and the time to the development of tumours in $\geq$90% of the animals. In addition, the percentage of animals with at least one large tumour ($\geq$10 mm^3) and the incidence of spontaneous remissions were evaluated for each carcinogenesis protocol over the follow-up period that was feasible in each case.

DNA synthesis was evaluated in tissue with PMD in terms of 5-bromo-2′-deoxyuridine (BrdU) incorporation at T0 for the 6- and 12-week protocols and at 8 months post-T0 for the 6-week protocol (animals treated with the 12-week protocol were not amenable to reliable follow-up after 30 days post-T0). Thirty minutes prior to humane killing we administered 2 ml of a 1% solution of BrdU in distilled water (approximately 0.2 g BrdU/kg body weight) intraperitoneally (i.p.) to each hamster. Tissue samples were removed and fixed in 10% buffered formaline, paraffin embedded, sectioned at 5 μm and processed for immunohistochemical demonstration of BrdU employing the peroxidase–antiperoxidase technique.[16] Following antigen retrieval with 0.1 M citrate buffer, pH 6, in a microwave oven, we performed BrdU detection using a mouse monoclonal anti-BrdU antibody (clon IIB5; Biogenex, San Francisco, CA, USA) and the biotin-streptavidine-peroxidase kit (Kit Multilink; Biogenex). Slides were counterstained with haematoxylin and mounted. Nuclei with positive staining exhibited brown diaminobenzidine deposits that contrasted against unlabeled, blue, haematoxylin-stained nuclei. BrdU-labeled nuclei were counted by light microscopy at 400× magnification as the brown nuclei in all the epithelial strata above a fixed length (300 μm) of basal layer employing a grid fitted into the eyepiece, spanning the full length of the basal layer available for each pouch section. Following the verification that the known histological lesions present in the tissue with PMD induced by the classical 12-week protocol were consistently present in the tissue with PMD induced by the 6-week protocol, the following 3 histological categories were evaluated individually for both protocols: No Unusual Microscopic Features (NUMF) or cancerized epithelium with no morphological lesions as a model of early field cancerization, hyperplasia and dysplasia.[15] Eight to fourteen animals were evaluated for each carcinogenesis protocol and time-point. One pouch section was measured for each hamster. All the areas corresponding to each of the histological categories represented in each pouch section were counted. The number of fields corresponding to each histological category varied

Table 1 – Number of topical applications of DMBA corresponding to each carcinogenesis protocol and the percentage of animals that developed tumours from tissue with PMD at T0 and at representative time-points post-T0, for each of the different carcinogenesis protocols.

Carcinogenesis protocol	Number of topical applications	Time				
		T0	1 month	2 months	3 months	8 months
12 weeks ($n = 19$)	36	90%	100%			
8 weeks ($n = 19$)	16	47%	90%	95%	100%	
7 weeks ($n = 19$)	14	32%	74%	79%	90%	
6 weeks ($n = 77$)	12	9%	65%	78%	87%	97% ($n = 38$)[a]
4 weeks ($n = 21$)	8	0%	19%	33%	38%	38%

[a] The difference in "n" is due to the fact that an additional series of animals treated with the 6-week carcinogenesis protocol was followed for 3 months to increase the sample size for the earlier time-points.

from pouch to pouch. Hence, the difference in the number of fields evaluated in each case.

Likewise, DNA synthesis was evaluated in tumour tissue (when available) adjacent to tissue with PMD at 8 months post-T0 for the 6-week protocol, and compared to values at T0 for the 12-week protocol previously reported by Aromando et al.[17] (at T0 the material available for histological analysis of tumours corresponding to the 6-week protocol was overly scarce and, as previously described, the 12-week protocol was not amenable to prolonged follow-up). Software adapted from a standard morphometric program was employed to count brown and blue stained nuclei. Ten random 255 μm $\times$ 185 μm fields were measured at 200$\times$ magnification on one section for each tumour. Approximately, 200 ± 100 tumour cells were contained in each evaluated field. A proliferative index (PI) was calculated as the percentage ratio between BrdU-positive cells and total cells counted. The values corresponding to normal (non-cancerized) pouch tissue were taken from a series of contralateral, non-cancerized, normal pouches that served as controls in a previous study by our group.[15] In all cases, adjacent, haematoxylin-eosin stained sections were employed for histological observation.

The statistical significance of the differences in DNA synthesis values between the different carcinogenesis protocols and follow-up times was evaluated by Student's unpaired t-test. The statistical significance of the differences in DNA synthesis values amongst the different histological categories of tissue with PMD was evaluated by one-way ANOVA. Overall level of significance was set at $p = 0.05$.

3. Results

Feasibility of follow-up varied with the aggressiveness of the carcinogenesis protocols. As described above, the 12-week protocol only allowed for a *short-term* follow-up (1 month post-T0) due to the rapid development of large tumours that impair feeding and compromise the quality of life of the animals. In addition, numerous DMBA applications would cause liver disorders such as enhanced oxidation of lipids and proteins coupled to compromised antioxidant defenses,[18] contributing to animal decline when long follow-up periods are attempted. Thus, the 12-week protocol is adequate to induce tumours for use in our *short-term* tumour control studies but is not amenable to *long-term* studies. Only the 4- and 6-week protocols allowed for consistently *long-term* follow-up (8 months post-T0). The 7- and 8-week protocols allowed for intermediate follow-up periods; in the present study follow-up was discontinued at 3 months post-T0.

Table 1 shows the number of topical applications of DMBA corresponding to each protocol (from 8 applications in the 4-week protocol to 36 applications in the 12-week protocol) and the percentage of animals that developed tumours from tissue with PMD at T0 and at representative time-points post-T0 for each of the carcinogenesis protocols. The percentage of animals bearing tumours increased progressively with the aggressiveness of the carcinogenesis protocols and time post-T0. T50 decreased with increasing aggressiveness of the carcinogenesis protocols, i.e. 21 days for the 6-week protocol, 14 days for the 7-week protocol and 3 days for the 8-week protocol. In the case of the 12-week protocol, 90% of the animals had already developed tumours by T0 whereas in the case of the 4-week protocol, T50 was not reached within the follow-up period of 8 months. The time at which tumours developed in $\geq$90% of the animals was at T0 for the 12-week protocol, 1 month post-T0 for the 8-week protocol, 3 months post-T0 for the 7-week protocol and 4 months post-T0 for the 6-week protocol. $<$40% of the animals in the 4-week protocol developed tumours within the 8 months follow-up period. The failure of the 4-week protocol to guarantee tumour development in the majority of the animals rendered it inadequate to give rise to a model of tissue with PMD to study the potential inhibitory effect of BNCT (or other therapeutic strategies) on the development of tumours.

Table 2 shows the percentage of animals with at least one large tumour (>10 mm^3). This end-point shows that the incidence of larger tumours increases with time and the aggressiveness of the carcinogenesis protocols.

Spontaneous remissions ranged from 6 to 20% for the 4-, 6- and 7-week protocols within the corresponding follow-up period. The 8- and 12-week protocols did not exhibit any spontaneous remissions within the corresponding follow-up period.

The histological analysis of the tissue with PMD induced by the 6-week protocol (Fig. 1) confirmed the existence of the same histological categories that are known to exist in the tissue with PMD induced by the classical 12-week protocol,[15] i.e. NUMF (no unusual microscopic features): an epithelium with no apparent lesions but with subepithelial fibrosis; hyperplasia; dysplasia. These areas coexist with tumours (Fig. 1A–C).

Table 2 – Percentage of animals available for evaluation with at least one large tumour (>10 mm^3) at representative time-points post-T0 for each of the different carcinogenesis protocols.

Carcinogenesis protocols	Time post-T0 (months)		
	1 month	3 months	8 months
12 weeks (n = 19)	100%	–	–
8 weeks (n = 19)	26%	79%	–
7 weeks (n = 19)	21%	26%	–
6 weeks (n = 77)	5%	27%	33% (n = 18)[a]
4 weeks (n = 21)	0%	0%	0%

[a] The difference in "n" is due to the fact that an additional series of animals treated with the 6-week carcinogenesis protocol was followed for 3 months to increase the sample size for the earlier time-points.

Table 3 shows the number of BrdU-labeled nuclei/field in each of the histological categories of tissue with PMD (NUMF, hyperplasia and dysplasia) for the 6-week carcinogenesis protocol at T0 and at 8 months post-T0, and for the 12-week carcinogenesis protocol at T0. Overall, BrdU-labeled nuclei increased progressively with time and protocol aggressiveness. The number of BrdU-labeled nuclei increased concomitantly with the histological aggressiveness of the lesion, i.e. NUMF < hyperplasia < dysplasia ($p < 0.0001$). Statistical analysis of the data showed that the values for the 6-week protocol at T0 were significantly lower than the corresponding values for the 12-week protocol at T0 ($p < 0.002$). Moreover, even at 8 months post-T0 they did not reach the T0 values of the 12-week protocol ($p < 0.03$). For the 6-week protocol, the values for 8 months post-T0 were significantly higher than for T0 in the case of hyperplasia and NUMF ($p < 0.0001$). The differences for dysplasia did not reach statistical significance ($p > 0.12$). In virtually all cases corresponding to cancerized tissue, the values were significantly higher ($p < 0.0001$) than for normal (non-cancerized) pouch tissue (0.81 ± 1.22 BrdU-labeled nuclei/field). The only exception was the value for NUMF at T0 for the 6-week protocol (0.7 ± 1.2 BrdU-labeled nuclei/field) that did not exhibit a statistically significant difference with the corresponding value for normal tissue ($p > 0.2$).

The proliferative index (PI) in tumour tissue was significantly ($p = 0.0001$) higher at T0 for the 12-week protocol ($18.0 \pm 5.8\%$, $n = 18$ tumours) than at 8 months post-T0 for the 6-week protocol ($6.0 \pm 4.1\%$, $n = 6$ tumours). A representative example of a tumour area corresponding to the 6-week carcinogenesis protocol and exhibiting BrdU-labeled nuclei is shown in Fig. 1D.

The 6-week protocol allowed for a *long-term* follow-up (8 months post-T0) and exhibited tumour development in ≥90% of the animals at 8 months.

4. Discussion

Control of tissue with PMD is pivotal in oncological therapy. Its clinical relevance lies in the development of carcinomas from multifocal areas of precancerous change,[13] leading to locoregional recurrences that frequently jeopardize therapeutic success.[11,15,19] Within this context, although in previous studies we achieved remarkable tumour control[5–8] with no normal tissue radiotoxicity in the hamster cheek pouch oral cancer model, we still face the challenge of achieving and demonstrating a *long-term* inhibitory effect on the development of tumours from tissue with PMD without exceeding the radiotolerance of this tissue. An adequate model to study the potential *long-term* therapeutic/toxic effects of BNCT or other therapeutic strategies was lacking.

Our goal was to develop a model of tissue with PMD in the hamster cheek pouch that would allow for *long-term* studies of therapeutic efficacy and toxicity. The carcinogenesis protocol should be sufficiently aggressive to guarantee tumour development post-completion of the carcinogenesis protocol in a vast majority of the animals within the follow-up period, but be sufficiently well-tolerated to allow for *long-term* follow-up.

We evaluated 5 potential carcinogenesis protocols in the hamster cheek pouch. Follow-up time was determined for each group based on the health status of the animals and the extent of feeding impairment caused by tumour development in the pouch. Tumour development after completion of the carcinogenesis protocol was assessed in terms of a series of quantitative parameters based on gross observations and measurements. Likewise, DNA synthesis was evaluated in tissue with PMD and tumour tissue for representative protocols and time-points.

The accumulation of repeated carcinogen applications in the hamster cheek pouch would conceivably lead to the

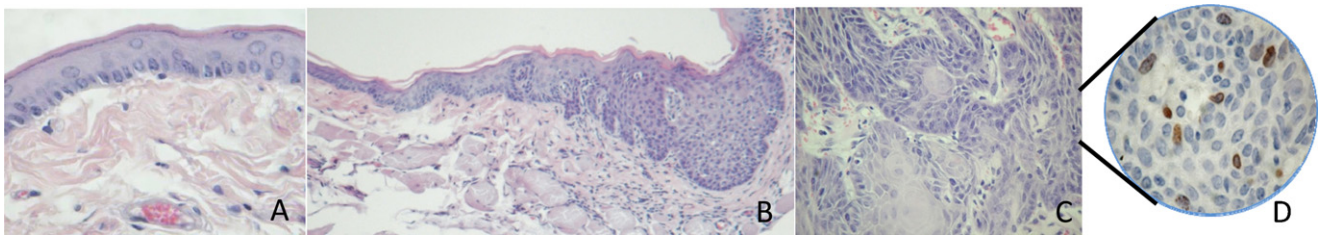

Fig. 1 – Representative examples of epithelial alterations corresponding to the 6-week carcinogenesis protocol. (A) Normal epithelium in a non-cancerized pouch (H&E, 400×); (B) Transition between NUMF (No Unusual Microscopic Features), hyperplastic and dysplastic epithelium in a cancerized pouch (H&E, 200×); (C) Tumour area exhibiting tumour cords with corneum differentiation and atypia (H&E, 400×); (D) Immunohistochemical labeling of BrdU in a tumour area. Brown, BrdU-positive nuclei in DNA synthesis contrast against BrdU-negative blue nuclei (1000×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 3 – Number of BrdU-labeled nuclei/field in normal (non-cancerized) epithelium and in each of the histological categories of tissue with PMD for the 6-week carcinogenesis protocol at T0 and 8 months post-T0, and for the 12-week carcinogenesis protocol at T0. The values are expressed as mean ± S.D. "*n*" indicates the total number of fields measured per protocol. NUMF: No Unusual Microscopic Features.

Carcinogenesis protocols	T0			8 months post-T0		
	Dysplasia	Hyperplasia	NUMF	Dysplasia	Hyperplasia	NUMF
12 weeks	18.0 ± 9.5 (*n* = 79)	9.2 ± 6.9 (*n* = 286)	4.0 ± 3.6 (*n* = 618)	–	–	–
6 weeks	5.2 ± 7.6 (*n* = 6)	1.6 ± 2.1 (*n* = 186)	0.7 ± 1.2 (*n* = 226)	10.6 ± 4.6 (*n* = 8)	3.7 ± 3.2 (*n* = 205)	1.7 ± 1.7 (*n* = 248)
Normal (non-cancerized) epithelium: 0.8 ± 1.2 (*n* = 758)						

progressive accumulation of genetic changes and development of microenvironmental cues and selection pressure.[20–22] As the number of carcinogen applications increases, the probability of malignant transformation rises. The present findings on feasibility of follow-up and tumour development evidence a clear correlation amongst animal decline, tumour yield and tumour size with the degree of aggressiveness of the carcinogenesis protocols. The more aggressive protocols lead to rapid tumour development, larger tumours, and the constraint of shorter follow-up periods. The incidence of tumour remissions was also related to the aggressiveness of the carcinogenesis protocols. The influence of spontaneous tumour regression, a rare but established phenomenon,[20] is taken into account in actual therapy/toxicity studies by comparison with the untreated control group. In addition, the values of DNA synthesis were higher for the more aggressive 12-week carcinogenesis protocol than for the less aggressive 6-week protocol. Even the 8 months values corresponding to the 6-week protocol were lower than the T0 values of the 12-week protocol. These findings illustrate how proliferative capacity contributes to tumour development. The only two protocols that enabled *long-term* follow-up (8 months) were the 4-week protocol and the 6-week protocol. However, the 4-week protocol did not guarantee tumour development in a vast majority of the animals within the follow-up period. Conversely, the 6-week protocol guaranteed tumour development in $\geq$90% of the animals within the follow-up period, a finding that was interpreted as proof of the induction of tissue with PMD, the pre-requisite. In a previous study, Feng and Wang[23] developed a precancer model in the hamster cheek pouch based on topical application of 0.5% DMBA 3 times a week for 5 weeks (15 applications). That study analyzed chemopreventive effects with only a 3.5 month follow-up. Typically, there is a significant increase of karyotypes demonstrating tetraploidy or near-tetraploidy by the second week of DMBA treatment.[24] By 5 weeks, cells become strongly angiogenic.[25] The 5-week DMBA treatment (application 3 times a week yielding a total of 15 applications) may be regarded as the precancerous stage of the oral carcinogenesis process.[23] The 6-week protocol (application twice a week yielding a total of 12 applications) developed herein lies within this range but allows for an 8 months follow-up.

The aim of the present study was not to evaluate the broad range of potential markers of precancer and cancer during the process of carcinogenesis or the potential mechanisms involved in the process of malignant transformation. Highly contributory studies have addressed these issues at different stages *during* the classical DMBA carcinogenesis protocol that involves topical application 3 times a week for 12–16 weeks.[4,25–34] However, information was lacking on actual tumour development and follow-up feasibility at different times *after* the completion of different carcinogenesis protocols in the hamster cheek pouch. The present comparative analysis of 5 carcinogenesis protocols for the longest possible reproducible follow-up period showed that 12 DMBA applications as in the 6-week protocol are enough to reliably induce tissue with PMD but preserve the animal and pouch enough to allow for *long-term* follow-up. If intermediate follow-up times were sufficient (3 months), the 7- and 8-week protocols would be adequate to evaluate more aggressive tissue with PMD, an issue of clinical relevance in terms of therapeutic effects and tolerance.

The 6-week protocol was selected to evaluate the *long-term* potential inhibitory effect of BNCT or other therapeutic modalities on the development of second primary tumours from tissue with PMD and assess concomitant potential *long-term* toxicity in this tissue as a dose-limiting tissue.

Acknowledgments

This work was partially supported by a grant from Agencia Nacional de Promoción Científica y Tecnológica of Argentina.

AMH, MEI, VAT and AES are members of the National Research Council (CONICET).

Funding: Grant of Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT2006–00700).

Competing interest: No conflict of interest to declare.

Ethical approval: The protocols employed in the present study have been examined and approved by an Institutional Review Board.

REFERENCES

1. Kastenbauer E, Wollenberg B. In search of new treatment methods for head and neck carcinoma. *Laryngol Rhinol Otol* 1999;**78**(1):31–5.

2. Salley JJ. Experimental carcinogenesis in the cheek pouch of the Syrian hamster. *J Dent Res* 1954;**33**:253–62.
3. Morris AL. Factors influencing experimental carcinogenesis in the hamster cheek pouch. *J Dent Res* 1961;**40**:3–15.
4. Vairaktaris E, Spyridonidou S, Papakosta V, Vylliotis A, Lazaris A, Perrea D, et al. The hamster model of sequential oral oncogenesis. *Oral Oncol* 2008;**44**(4):315–24.
5. Kreimann EL, Itoiz ME, Longhino J, Blaumann H, Calzetta O, Schwint AE. Boron neutron capture therapy for the treatment of oral cancer in the hamster cheek pouch model. *Cancer Res (Advances in Brief)* 2001;**61**:8638–42.
6. Trivillin VA, Heber EM, Itoiz ME, Nigg D, Calzetta O, Blaumann H, et al. Radiobiology of BNCT mediated by GB-10 and GB-10 + BPA in experimental oral cancer. *Appl Radiat Isot* 2004;**61**:939–45.
7. Trivillin VA, Heber EM, Nigg DW, Itoiz ME, Calzetta O, Blaumann H, et al. Therapeutic success of Boron Neutron Capture Therapy (BNCT) mediated by a chemically non-selective boron agent in an experimental model of oral cancer: a new paradigm in BNCT radiobiology. *Radiat Res* 2006;**166**:387–96.
8. Pozzi E, Nigg DW, Miller M, Thorp SI, Heber EM, Zarza L, et al. Dosimetry and radiobiology at the new RA-3 reactor boron neutron capture therapy (BNCT) facility: application to the treatment of experimental oral cancer. *Appl Radiat Isotopes* 2009;**67**:309–12.
9. Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. *J Oral Pathol Med* 2007; **36**:575–80.
10. Napier SS, Speight PM. Natural history of potentially malignant oral lesions and conditions: an overview of the literature. *J Oral Pathol Med* 2008;**37**:1–10.
11. Smith BD, Haffty BG. Molecular markers as prognostic factors for local recurrence and radioresistance in head and neck squamous cell carcinoma. *Radiat Oncol Investig* 1999;**7**(3):125–44.
12. Schwint AE, Savino TM, Lanfranchi HE, Marschoff E, Cabrini RL, Itoiz ME. Nucleolar organizer regions in lining epithelium adjacent to squamous cell carcinoma of human oral mucosa. *Cancer* 1994;**73**:2674–9.
13. Braakhuis BJM, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization. *Cancer Res* 2003;**63**:1727–30.
14. Barth RF, Coderre JA, Vicente MGH, Blue TE. Boron neutron capture therapy of cancer: current status and future prospects. *Clin Cancer Res* 2005;**11**:3987–4002.
15. Heber EM, Aromando RF, Trivillin VA, Itoiz ME, Nigg DW, Kreimann EL, et al. Therapeutic effect of boron neutron capture therapy (BNCT) on field cancerized tissue: inhibition of DNA synthesis and lag in the development of second primary tumors in precancerous tissue around treated tumors in DMBA-induced carcinogenesis in the hamster cheek pouch oral cancer model. *Arch Oral Biol* 2007;**52**:273–9.
16. Sternberg LA, Hardy PH, Cuculis JJ, Meyer HG. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen–antibody complex (horseradish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem* 1970;**18**:315–30.
17. Aromando RF, Heber EM, Trivillin VA, Nigg DW, Schwint AE, Itoiz ME. Insight into the mechanisms underlying tumor response to boron neutron capture therapy in the hamster cheek pouch oral cancer model. *J Oral Pathol Med* 2009;**38**(5):448–54.
18. Letchoumy PV, Chandra Mohan KV, Kumaraguruparan R, Hara Y, Nagini S. Black tea polyphenols protect against 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. *Oncol Res* 2006;**16**(4):167–78.
19. Kankaanranta L, Seppälä T, Koivunoro H, Saarilahti K, Atula T, Collan J, et al. Boron neutron capture therapy in the treatment of locally recurred head and neck cancer. *Int J Radiat Oncol Biol Phys* 2007;**69**(2):475–82.
20. Baker SG, Kramer BS. Paradoxes in carcinogenesis: new opportunities for research directions. *BMC Cancer* 2007;**7**:151.
21. Laconi E, Sonnenschein C. Cancer development at tissue level. *Semin Cancer Biol* 2008;**18**(5):322–9.
22. Laconi E, Doratiotto S, Vineis P. The microenvironments of multistage carcinogenesis. *Semin Cancer Biol* 2008;**18**(5): 322–9.
23. Feng L, Wang Z. Chemopreventive effect of celecoxib in oral precancers and cancers. *Laryngoscope* 2006;**116**(10):1842–5.
24. Lin MH, Hsieh SY, Li SY, Shih HC, Chiang J, McBride J, et al. Sequential cytogenetic alterations in hamster oral keratinocytes during DMBA-induced oral carcinogenesis. *Eur J Cancer Oral Oncol* 1994;**30B**(4):252–64.
25. Lingen MW, DiPietro LA, Solt DB, Bouck NP, Polverini PJ. The angiogenic switch in hamster buccal pouch keratinocytes is dependent on TGFbeta-1 and is unaffected by ras activation. *Carcinogenesis* 1997;**18**(2):329–38.
26. Nagini S, Letchoumy PV, Thangavelu A, Ramachandran CR. Of humans and hamsters: a comparative evaluation of carcinogen activation, DNA damage, cell proliferation, apoptosis, invasion, and angiogenesis in oral cancer patients and hamster buccal pouch carcinomas. *Oral Oncol* 2009;**45**:e31–7.
27. Chang KW, Lin SC, Koos S, Pather K, Solt D. p53 and Ha-ras mutations in chemically induced hamster buccal pouch carcinomas. *Carcinogenesis* 1996;**17**(3):595–600.
28. Gimenez-Conti IB, Shin DM, Bianchi AB, Roop DR, Hong WK, Conti CJ, et al. Changes in keratin expression during 7,12-dimethylbenz(a)anthracene-induced hamster cheek pouch carcinogenesis. *Cancer Res* 1990;**50**(14): 4441–5.
29. Shin DM, Gimenez IB, Lee JS, Nishioka K, Wargovich MJ, Thacher S, et al. Expression of epidermal growth factor receptor, polyamine levels, ornithine decarboxylase activity, micronuclei, and transglutaminase I in a 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis model. *Can Res* 1990;**50**(8):2505–10.
30. Gimenez-Conti IB, Slaga TJ. The hamster cheek pouch carcinogenesis model. *J Cell Biochem Suppl* 1993;**17F**:83–90.
31. Gimenez-Conti IB, LaBate M, Liu F, Osterndorff E. P53 alterations in chemically induced hamster cheek-pouch lesions. *Mol Carcinog* 1996;**16**(4):197–202.
32. Bhuvaneswari V, Rao KS, Nagini S. Altered expression of anti and proapoptotic proteins during chemoprevention of hamster buccal pouch carcinogenesis by tomato and garlic combination. *Clin Chim Acta* 2004;**350**(1–2):65–72.
33. Raimondi A, Cabrini R, Itoiz ME. Ploidy analysis of field cancerization and cancer development in the hamster cheek pouch carcinogenesis model. *J Oral Pathol Med* 2005;**34**(4):227–31.
34. Hsue SS, Wang WC, Chen YK, Lin LM. Expression of inhibitors of apoptosis family protein in 7,12-dimethylbenz(a)anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis is associated with mutant p53 accumulation and epigenetic changes. *Int J Exp Pathol* 2008;**89**(5):309–20.