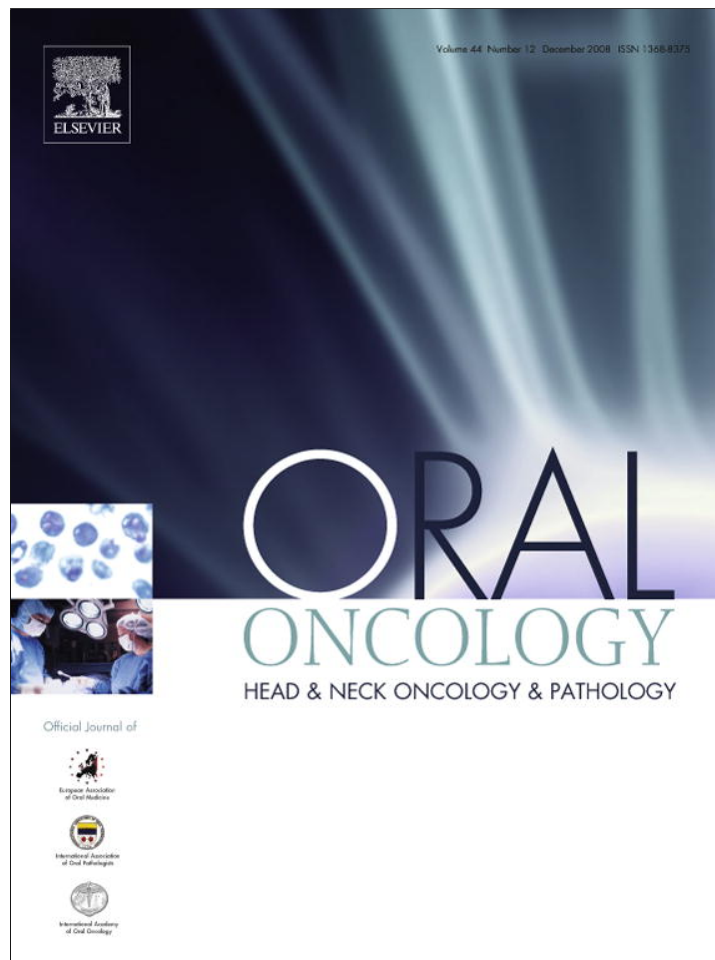


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The therapeutic effect of fractionated radiation on DMBA-induced hamster buccal pouch squamous cell carcinomas

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Summary Seventy hamsters were divided equally into experimental groups A and B and control groups C–G. After treating the pouches of groups A and B animals with DMBA (thrice a week) for 14 weeks, the heads of the animals received fractionated radiation of a total dose of 21Gy and 42Gy, respectively. The untreated pouches of groups C and D animals were similarly irradiated. The pouches of groups E and F animals were treated with DMBA or mineral oil for 14 weeks, respectively. The pouches of group G animals remained untreated throughout the experiment. Radiation response (RR) was not noted for 12 exophytic tumors of group A; the remaining 43 tumors showed partial response. For group B, no RR was noted for four exophytic lesions; the remaining 28 lesions revealed a combination of partial and complete response. No endophytic lesions of group A showed RR; a significant increase in radiation response was noted for group B compared with group A. In conclusion, the hamster pouch oral cancer model could be employed to study the effect of fractionated radiation.

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Introduction

Radiotherapy is a common treatment modality for head and neck cancer patients. To date, understanding of the exact biological mechanism of radiation oncology on oral cancer remains incomplete. In a review of literature concerning the therapeutic effects of radiation on oral cancer, it was noted that, apart from the retrospective clinical studies,^{1,2} most

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have been *in vitro* studies using human oral cancer cell lines directly³ or, indirectly, inoculating the cancer cells into nude mice, which subsequently receive irradiation treatment.³ However, incubation and viability of the *in vitro* cell lines are not easy to control; furthermore, the successors may not be completely identical to their parent cells. When using an animal model, the effects of radiotherapy that simulate the human treatment regime on experimentally induced oral cancer can be observed. The data obtained from animal studies are expected to be able to be translated to future human studies. Therefore, if an animal model could be established, it would be able to be used in future studies on the molecular mechanisms of radiotherapy.

Hamster buccal pouch mucosa constitutes one of the most widely accepted experimental models for oral carcinogenesis.⁴ Despite anatomic and histologic variations between hamster pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that resemble those that occur during analogous development in human oral mucosa.⁵ In the 1900s, the enhancing effect of 7,12-dimethyl[a]anthracene (DMBA) carcinogenesis on hamster buccal pouch mucosa by repeated exposure to low-level X radiation was extensively studied.^{6–8} The therapeutic effect of fractionated radiation on hamster buccal pouch carcinoma after induction has not been investigated, to our knowledge. The aim of the present study is therefore to determine the potential therapeutic effects of fractionated radiation on DMBA-induced hamster buccal pouch squamous cell carcinomas.

Materials and methods

Animals and treatments

Outbred, young (6-week-old), male Syrian golden hamsters (*Mesocricetus auratus*; 70 animals, purchased from the National Science Council Animal Breeding Center, Taipei, ROC), weighing approximately 100 g at the beginning of the experiment, were randomly divided into two experimental groups, A and B, and six control groups, C–G (Table 1). The animals were housed under constant conditions (22 °C, 12-h light/dark cycle) and supplied with tap water and standard Purina laboratory chow *ad libitum*. Appropriate animal care and an approved experimental protocol ensured humane treatment, and all procedures were conducted in accordance with the NIH *Guide for the Care and Use of Animals*.

After allowing the animals one week of acclimatization to their new surroundings, both pouches of the 20 animals in groups A and B were painted with 0.5% DMBA solution (wt/vol) using a No. 4 sable-hair brush at 9 a.m. every Monday, Wednesday and Friday, for 14 weeks. Approximately 0.2 ml of the appropriate solution was applied topically to the medial walls of both pouches at each painting. Bilateral pouches from the animals of two control groups were treated for 14 weeks with DMBA (group E) and mineral oil (group F), respectively. All 10 animals of the other control groups C, D and G remained untreated throughout the experiment.

Fractionated radiation regimen

The fractionated radiation regimen was implemented three days after the final painting. The animals were anesthetized with sodium pentobarbital and transported to the radiation oncology treatment area. The whole bodies of 10 animals in group A were placed within custom-made acrylic containers constructed to expose the head only; the remainder of the animal was protected with a 5-cm lead shield (Fig. 1). Subsequently, only the heads received fractionated radiation, with a total radiation dose of 21Gy (6 MV, 7Gy/twice/week)^{9–11} using a linear accelerator (Varian, 2100C, Palo Alto, CA, USA) with a field of radiation of 4 cm. The animals of group B were similarly irradiated, with a total radiation dose of 42Gy. The animals of the two control groups C and D (untreated throughout the experiment) were also similarly irradiated, with a total radiation dose of 21Gy and 42Gy, respectively.

Specimen collection

At 14 weeks, the animals of the three control groups E–G were killed simultaneously by administration of a lethal dose of diethyl ether, at 9 a.m., to avoid any influence of diurnal variation.¹² Their pouches were exposed by dissection and examined grossly; both pouches were then excised and placed on cardboard to prevent distortion of the pouch tissues. The number of growths was counted and the diameters of tumors were measured. The entire pouches were then serially sectioned and routinely processed for light microscopy by being fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in a series of ascending-concentration alcohol solutions, cleaned in xylene, and embedded in paraffin for hematoxylin–eosin staining. Three

Table 1 Treatment protocol, average tumor number and dimension (mm) of each group observed grossly

Group	Average tumor no.	Average tumor dimension (mm)
A: DMBA treatment (14 weeks) → fractionated radiation (21Gy)	5.7 ± 1.5 ^{a,*}	10.7 ± 2.4 ^{a,*}
B: DMBA treatment (14 weeks) → fractionated radiation (42Gy)	3.2 ± 0.8 ^{**}	6.3 ± 1.9 ^{**}
C: no treatment (14 weeks) → fractionated radiation (21Gy)	0.0 ± 0.0	0.0 ± 0.0
D: no treatment (14 weeks) → fractionated radiation (42Gy)	0.0 ± 0.0	0.0 ± 0.0
E: DMBA treatment (14 weeks)	6.3 ± 1.1	12.3 ± 2.5
F: mineral oil treatment (14 weeks)	0.0 ± 0.0	0.0 ± 0.0
G: no treatment (14 weeks)	0.0 ± 0.0	0.0 ± 0.0

^a Means ± standard deviation.

^{**} $p < 0.05$, compared with group E.

^{*} $p > 0.05$, compared with group E.

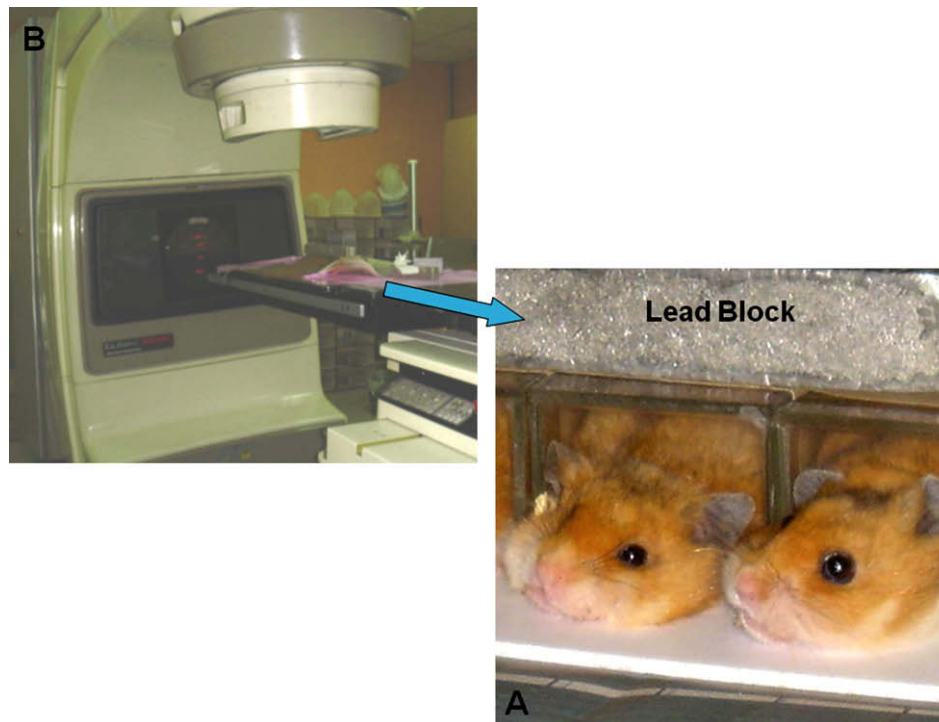


Figure 1 Body of the hamster was kept within a custom-made acrylic container protected with lead; only the head of the animal was exposed to radiation (A) with a linear accelerator (6 MV/7Gy/twice/week) (B).

weeks after completion of the final course of fractionated radiation,¹¹ the animals from the two experimental groups, A and B, as well as the control groups C and D, were similarly killed and the pouch tissues handled as described above.

Radiation response score evaluation

In accordance to our previous study,¹³ upon DMBA treatment, hyperkeratosis was noted microscopically in the 3-week DMBA-treated pouches and areas of epithelial hyperplasia were observed in the 7-week DMBA-treated pouches. Finally, both exophytic and infiltrative endophytic squamous cell carcinomas (SCCs) were detected in the 14-week DMBA-treated mucosa in the current experiment.

The irradiation effect of tissue necrosis of the induced tumor (both exophytic and endophytic) could be microscopically observed distinct from the adjacent pouch mucosa as the contiguous mucosa did not have apparent tissue damage upon the fractionated radiation protocol used in current experiment. The radiation response score of each exophytic and endophytic lesion for the whole pouch mucosa was then assessed by grading microscopically using a 5-point system: no radiation response on tumor cells – '0'; percentage of radiation response on tumor cells – <10%: '1'; 11%–50%: '2'; 51%–80%: '3'; 81%–100%: '4'. The total scores were calculated by summation of the individual radiation response score for each exophytic and endophytic lesion, respectively, of the entire pouch mucosa. For example, a pouch tissue contained three exophytic lesions with radiation response scores of 2, 2 and 3, and two endophytic lesions with radiation response scores of 1 and 1. The total score of the exophytic lesions for this pouch tissue was then calculated as: $(2 + 2 + 3) = 7$ whereas that of the endophytic

lesion was enumerated as: $(1 + 1) = 2$. Finally, the average score of the exophytic lesions was calculated by dividing the total score of the exophytic lesions with the total number of induced exophytic tumors with various degrees of radiation responses. Similarly, the average score of the endophytic lesions was calculated by dividing the total score of the endophytic lesions with the total number of induced endophytic tumors with various degrees of radiation responses. The radiation response score of each lesion was evaluated by two experienced oral and maxillofacial pathologists (Chen and Wang), who independently evaluated the radiation scores. When disagreement existed amongst the two observers, a consensus was reached by discussion. Interobserver agreement was evaluated using kappa statistics.¹⁴ The kappa value was calculated to assess interobserver agreement. A kappa value of less than 0.40 was considered as showing poor agreement; one of 0.40–0.59, fair agreement; one of 0.60–0.74, good agreement; and one of 0.75–1.00, excellent agreement.

Statistical analysis

Statistical analyses were examined by ANOVA using the SAS software package (SAS Institute Inc., Cary, NC). *p* values <0.05 were considered as significant.

Results

Gross observation (Table 1)

Upon gross observation, the average tumor number and dimension of pouches of group A (Fig. 2A) were less than

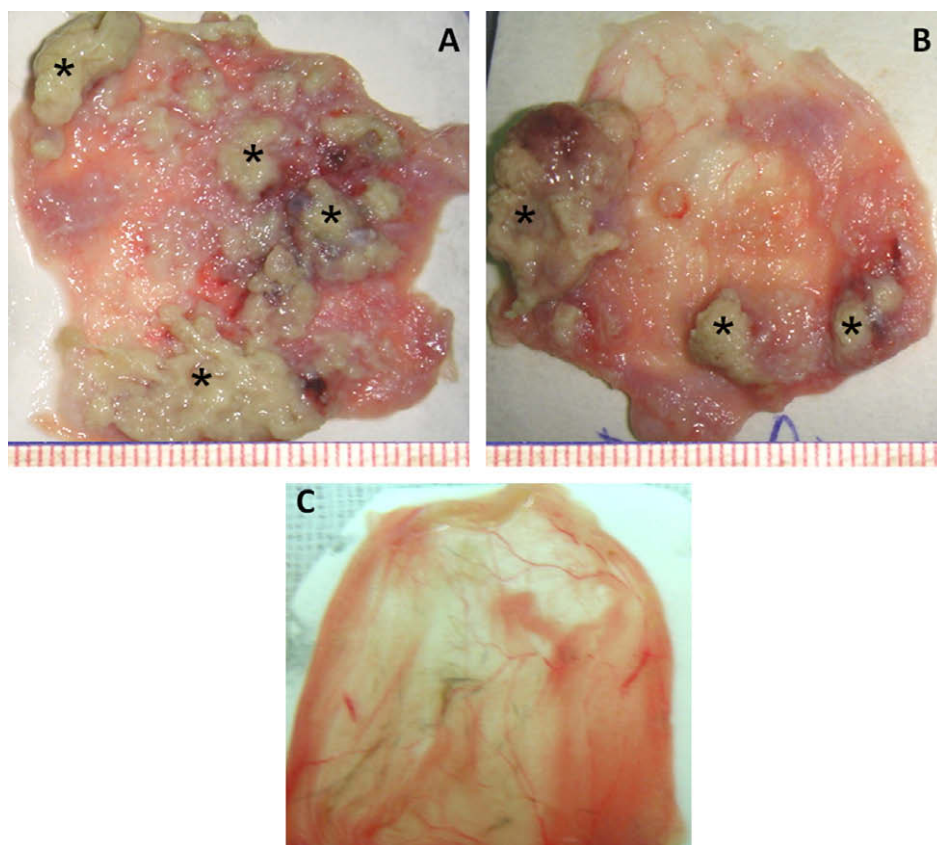


Figure 2 Representative samples of macroscopic tumors of pouches irradiated with a total radiation dose of 21Gy (A) or 42Gy (B) (* typical sites of yellowish tissue necrosis due to irradiation; scale bar: one space equivalent to 1 mm). Representative sample of pouches without DMBA treatment irradiated with a total radiation dose of 42Gy (C).

those of group E; however, the differences were not statistically significant. In contrast, both the average tumor number and dimension of pouches of group B (Fig. 2B) were significantly less than those of group E (Fig. 3). The pouches of groups C and D were grossly flat and tumor-free, with thickened mucosa (Fig. 2C). In addition, gross examination of the pouches of both the mineral oil-treated and untreated groups (F and G) revealed no obvious changes.

Histologic observation

Microscopically, as compared with groups E and F, the pouches of groups A and B showed hyalinized lamina propria, muscle atrophy and thickened blood vessel walls. Not only the histologic evidence of well-differentiated exophytic SCC (Fig. 4A) but also moderately differentiated endophytic lesions (Fig. 4B) were noted in the pouches of groups A, B and E; there were more exophytic lesions than endophytic ones. Consistent with macroscopic examination, the total tumor number of microscopic exophytic lesions of group E was slightly higher than that of group A and was significantly higher than that of group B. The total number of endophytic lesions of group E was slightly higher than both of groups A and B. Mucositis was noted in all pouches in groups A, B and E. On the other hand, no obvious histologic changes were noticed in any of the pouches of groups C and D or groups F and G.

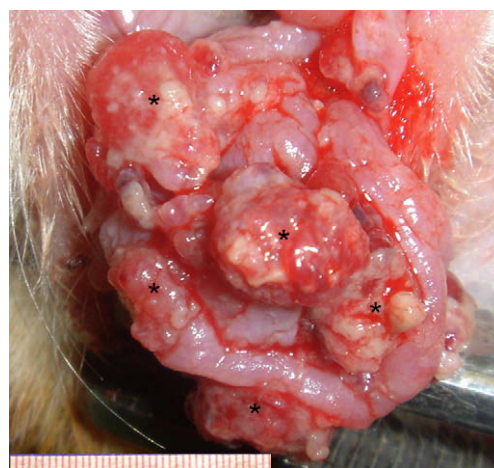


Figure 3 Representative sample of macroscopic tumors of pouches treated for 14 weeks with DMBA without irradiation (* typical sites of exophytic tumors-induced by DMBA; scale bar: one space equivalent to 1 mm).

Radiation response score (Table 2)

Interobserver agreement amongst the two observers was excellent for the assessment of radiation scores, showing a kappa value of 0.91. Radiation response was not noted

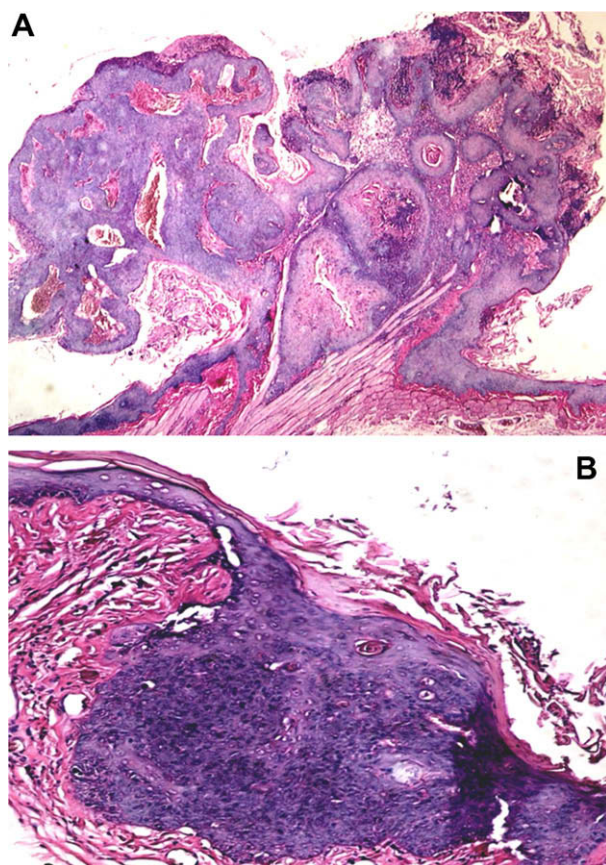


Figure 4 Representative sample of microscopic exophytic squamous cell carcinoma (A) and endophytic lesion (B) (hematoxylin and eosin stain 40 \times).

for 12 exophytic SCCs of group A, whilst the radiation response scores of the remaining 43 tumors were chiefly graded as 1 or 2 (Fig. 5A). No radiation response was noted for nine exophytic SCCs of group B; the remaining 28 tumors showed radiation response scores predominantly of grades 3 and 4, with eight tumors showing almost 100% response to irradiation (Fig. 5B). The average score of the exophytic lesions of group B was significantly higher than that of group A. It is of note that none of the endophytic lesions of group

A showed a response to radiation. Four endophytic lesions of group B showed no radiation response, with the radiation response scores of the remaining six lesions in the main graded at 2 or 3 (Fig. 5C). A significant increase in the average radiation response score of endophytic lesions was noted for group B as compared with group A.

Discussion

Single, whole-body doses (7–15Gy) of irradiation are commonly used in rodent models of radiation-induced salivary gland injury.¹⁰ Sonis et al.⁹ reported the occurrence of radiation mucositis on hamster cheek pouch mucosa when using a single high radiation dose of 35Gy, with the peak occurrence in 12–18 days after irradiation. Furthermore, Horn et al.¹¹ used a single dose of 20 Gy, which produced an absence of tumors in most of the hamster pouches treated with DMBA for 12 weeks, indicating radiogenic destruction of tumors. Taking these findings together, in this study, we used a fractionated dose of 7Gy (twice per week) with a total dose of 21Gy and then accumulated to 42Gy in order to demonstrate the therapeutic effect of a fractionated radiation regimen in a hamster cheek pouch oral cancer model.

Horn et al.¹¹ demonstrated the short (14 days) and long (39 days) term therapeutic effects on hamster cheek pouch carcinoma of using a single high radiation dose. They found that, in short term observation, there was radiogenic tumor destruction in most hamster cheek pouch carcinomas after induction; however, the occurrence of tumors in most hamsters after long term observation indicated that some vital tumor cells still existed post-irradiation and were sufficient to cause the reappearance of macroscopic tumors at a later period. Therefore, they suggested that in studies of the radiogenic cell-killing effect on experimental tumors, the observation period after irradiation should be long enough to preclude the likelihood of tumor recurrence after preliminary suppression. Nevertheless, we feared that after such a long observation period of up to 39 days, as demonstrated by Horn et al.,¹¹ some high cancer-bearing animals would have expired prior to the planned schedule of sacrifice. Hence, in the current study, we elected to kill the animals at 21 days after the final irradiation. Furthermore, as the average tumor number of both groups A and B was lesser

Table 2 Total number of exophytic and endophytic tumors observed microscopically as well as the average scores of the radiation response of those groups receiving fractionated radiation

Group	Total number of exophytic tumors	Average score ^a	Total number of endophytic tumors	Average score ^a
A	55 (43, 78.2%)	1.70 \pm 0.46	14 (0, 0%)	0.00 \pm 0.00
B	32 ^{**} (28, 87.5%)	3.29 \pm 0.46 [*]	10 (6, 60%)	2.50 \pm 0.55 [*]
C	0	0.00 \pm 0.00	0	0.00 \pm 0.00
D	0	0.00 \pm 0.00	0	0.0 \pm 0.00
E	61	NA	16	NA
F	0	NA	0	NA
G	0	NA	0	NA

NA: not applicable; values in parentheses indicate tumor number and percentage showing a response to radiation.

^a Means \pm standard deviation.

^{*} $p < 0.05$, compared with group A.

^{**} $p < 0.05$, compared with group E.

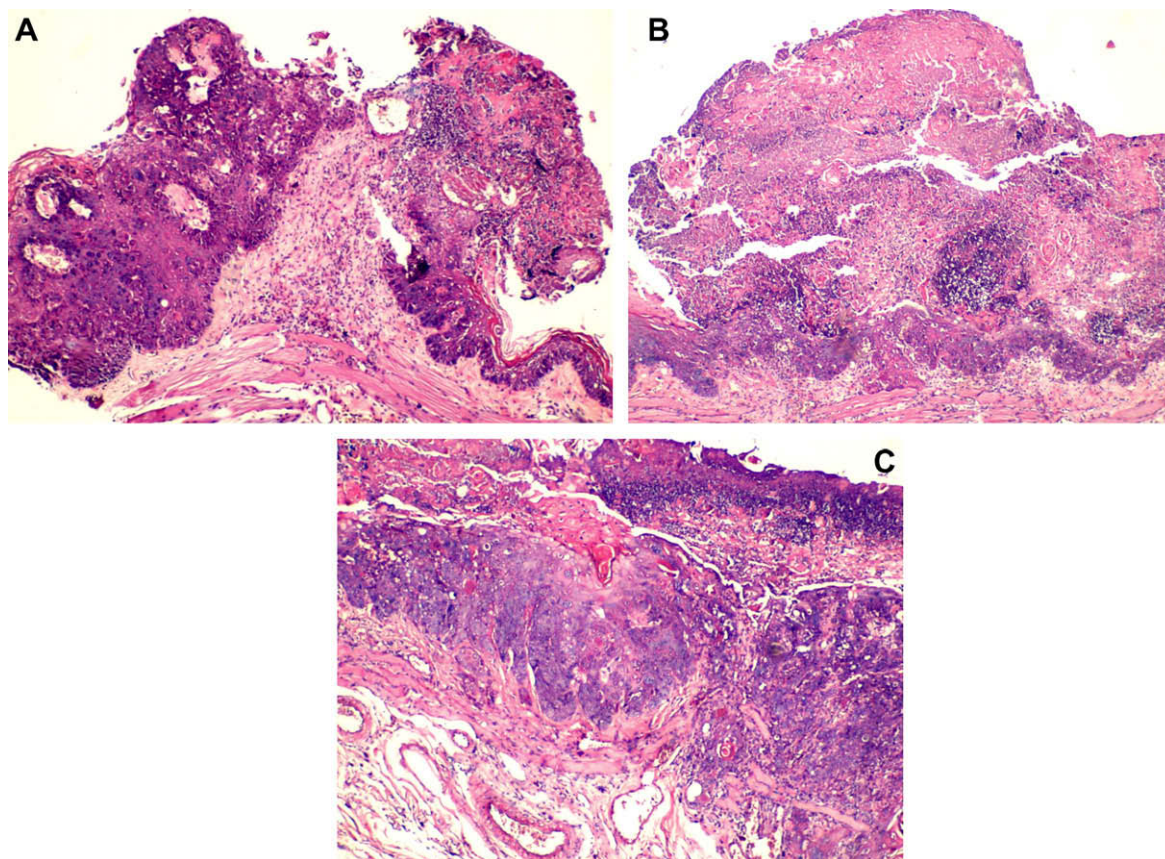


Figure 5 (A) Representative sample of an exophytic tumor irradiated with a total radiation dose of 21Gy showing a response score of grade 2 (hematoxylin and eosin stain 100 \times); (B) representative sample of an exophytic tumor irradiated with a total radiation dose of 42Gy showing a response score of grade 4 (hematoxylin and eosin stain 100 \times); (C) representative sample of an endophytic tumor irradiated with a total radiation dose of 42Gy showing a response score of grade 2 (hematoxylin and eosin stain 100 \times).

than group E, it might imply that no obvious tumor recurrence was found 21 days after irradiation. On the other hand, as the bilateral pouches of groups A–D required irradiation, whole head irradiation has the advantage that both pouches are irradiated simultaneously without the need to evert, secure and irradiate each pouch individually.

Animals such as mice, rats, hamsters, monkeys, guinea pigs and rabbits have been previously used to study the effects of radiation on mucosa and salivary glands.^{9,10,15–19} However, all of these studies used healthy animals without cancer. Moreover, the radiation modality used in these aforementioned studies^{9,10,15–19} was single irradiation, which differs from the routine clinical regimen of fractionated radiotherapy. Reviewing the literature, it can be seen that the application of a fractionated radiation regimen in animal studies has seldom been reported.^{10,20} Veninga et al.²⁰ studied the fractionated radiation effect on the transplanted rat rhabdomyosarcoma into the flank of rat, and Radfar and Sirois¹⁰ investigated the effect of radiation on minipig salivary glands using a fractionated radiation regimen. As mentioned above, the therapeutic response of hamster pouch carcinoma after induction by DMBA has only been reported when using single high dose irradiation.¹¹ To our knowledge, there have been no previous reports describing the therapeutic application of fractionated radiation for hamster pouch carcinoma after induction. As dem-

onstrated in the present study, hamster cheek pouch mucosa would be a reproducible and reliable animal model for studying the effect of fractionated radiation on oral cancer.

We found that, compared with the DMBA-treated pouches without irradiation (group E), with a total radiation dose of 21Gy (group A), there was no significant decrease in tumor number and dimension. This finding was compatible with the previous report of Horn et al.,¹¹ who irradiated the hamster carcinoma after induction with DMBA with a single dose of 21Gy. However, when the total dose was doubled (group B), both tumor number and dimension were significantly decreased. Furthermore, a significant increase in radiation response score of both exophytic and endophytic lesions was noted for group B as compared with group A. All these findings implied that a fractionated radiation regimen used in the hamster cheek pouch oral cancer model could simulate the radiotherapy treatment course of human oral cancer.

As a matter of fact, the lesser the differentiation, the more radiation-sensitive the irradiated tissues will be. Interestingly, the radiation response of the well-differentiated exophytic SCC was higher than that of the moderately differentiated endophytic lesion in this animal model. Therefore, difference in histologic differentiation between these two types of lesion would not be the determinant fac-

tor in explaining the discrepancy of radiation response in the hamster cheek pouch oral cancer model. Whether species difference or other molecular determinants contribute to such a disparity in radiotherapy treatment response warrants further investigation.

Although radiation mucositis was not the object of this study, we did observe an interesting phenomenon. In contrast to the findings of Sonis et al.,⁹ radiation mucositis did not occur in the irradiated pouches that did not receive DMBA treatment (groups C and D), implying that radiation mucositis in the hamster pouch would subside when using fractionated radiation instead of a single high dose of radiation.

In conclusion, due to the impaired healing capacity of irradiated human oral tissues, patients' samples would not be suitable for surgically intervened tissue examination. Animal studies would then be appropriate to investigate the effects of radiotherapy on oral cancer. In this study, we successfully demonstrated that the hamster cheek pouch oral cancer model would be appropriate for further investigations on the therapeutic effect of fractionated radiation.

Conflict of interest statement

None declared.

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