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OPTIMIZATION OF THE CLASSICAL ORAL CANCERIZATION PROTOCOL IN HAMSTER TO STUDY ORAL CANCER THERAPY

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Abstract

Objective(s): The hamster carcinogenesis model recapitulates oral oncogenesis. Dimethylbenz[a]anthracene (DMBA) cancerization induces early severe mucositis, affecting animal´s welfare and causing tissue loss and pouch shortening. "Short" pouches cannot be everted for local irradiation for Boron Neutron Capture Therapy (BNCT). Our aim was to optimize the DMBA classical cancerization protocol to avoid severe mucositis, without affecting tumor development. We evaluated BNCT in animals cancerized with this novel protocol. Materials and Methods: We studied: Classical cancerization protocol (24 applications); Classical with two interruptions (completed at the end of the cancerization protocol). BNCT mediated by boronophenylalanine (BPA) was performed in both groups. Results: The twice-interrupted group exhibited a significantly lower percentage of animals with severe mucositis vs the non-interrupted group (17% vs 71%) and a significantly higher incidence of long pouches (100% vs 53%). Tumor development and the histologic characteristics of tumor and precancerous tissue were not affected by the interruptions. For both groups, overall tumor response was more than 80%, with a similar incidence of BNCT-induced severe mucositis. Conclusion(s): The twice-interrupted protocol reduced severe mucositis during cancerization without affecting tumor development. This favoured the animal's welfare and reduced the number of animals to be cancerized for our studies, without affecting BNCT response.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide, which arises from the mucosa of the upper aerodigestive tract (Jimenez, Jayakar, Ow, Segall 2015; El-Bayoumy et al. 2017). The most frequent tumor sites are the larynx, the pharynx and the oral cavity, being the main risk factors consumption of alcohol, tobacco and the oncogenic HPV infection (Machiels et al. 2014). Several advances have been made in molecular diagnostics and therapeutics related to this illness. However, the 5-year survival rate continues to be one of the lowest of the major cancers (Mehritra, Ibrahim, Eckardt, Driemel, Singh 2011; Nagini & Kowshik, 2016).

Various animal models have been developed for studying pathogenesis, genetic background and development of novel therapeutic approaches in HNSCC. There are several animal models of oral cancer, such as xenograft, transgenic and chemically-induced animal models. In particular, in the case of the chemically-induced models, several agents have been assayed including coal tar, tobacco smoke constituents such us the dibenzo[a,l]pyrene (DB[a,l]P), the synthetic water-soluble 4-nitroquinoline1-oxide (4-NQO), 3-methylcholanthrene, and 7, 12-dimethylbenz(a)anthracene (DMBA) (Mognetti, Di Carlo, Berta 2006; El-Bayoumy et al. 2017).

Within the context of the chemically-induced animal models, the hamster model is a time-honored animal model used for the study of carcinogenesis, cancer prevention (Supsavhad, Dirksen, Martin, Rosol 2016) and treatment in the field of HNSCC (Monti Hughes et al. 2013, 2017). It was first developed in 1954 by Salley (1954), and then standardized by Morris (1961) and Shklar (1972). The golden hamster, Mesocricetus auratus, has an anatomic feature that resembles a pocket within the thickness of each cheek, which is lined with a stratified squamous epithelium resembling the oral cavity. This pouch is easily everted due to the loose adventitious tissue that separates the cheek mucosa from the skin. This allows a macroscopic follow up of the tissue (Monti Hughes et al. 2015a; Nagini & Kowshik 2016) and certain experimental procedures such as wound induction (e.g. Perez, Raimondi, Itoiz 2005) or local irradiation (e.g. Pozzi et al. 2009). Besides, as the pouch is easily accessible in situ, it is not necessary to anesthetise the animals during oral carcinogenesis.

The cancerization protocol in the hamster cheek pouch consists of the topical application of subthreshold doses of the complete carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), a prototype of the polycyclic aromatic hydrocarbons involved in the development of human oral cancer (Monti Hughes et al. 2015a; Nagini & Kowshik 2016). This model allows for the study of

tumors and the precancerous tissue around tumors, mimicking the spontaneous process of malignant transformation. In the same treated pouch, different stages of evolution of the carcinogenesis process can coexist, i.e. epithelium with no unusual microscopic features (NUMF), hyperplasia, dysplasia, exophytic and endophytic tumors (Heber et al. 2007). The most visible tumors are exophytic carcinomas, which can be objectively measured in everted pouches and exhibit similarities to human oral squamous cell carcinoma (OSCC) in terms of morphology, histology, pre-neoplastic lesions, expression of biochemical and molecular markers, and genetic and epigenetic alterations (Monti Hughes et al. 2015a; Nagini & Kowshik 2016).

The hamster cheek pouch model of oral cancer was proposed and validated by our group for boron neutron capture therapy (BNCT) studies (e.g. Kreimann et al. 2001; Trivillin et al. 2006; Pozzi et al. 2009; Molinari et al. 2012; Heber et al. 2014). Boron neutron capture therapy (BNCT) combines the administration of boron carriers that are taken up preferentially by neoplastic tissue and irradiation with a thermal/epithermal neutron beam. The capture of a thermal neutron by a ^{10}B nucleus results in the emission of high linear energy transfer (LET) α particles and recoiling ^{7}Li nuclei, both particles with high relative biological effectiveness. Their short range in tissue (6–10 μm) would limit the damage largely to cells containing ^{10}B, targeting the neoplastic tissue selectively, sparing normal tissue (Trivillin et al. 2006). BNCT clinical trials, employing nuclear reactors as neutron sources, were performed or are underway in USA, Japan, Taiwan, Europe and Argentina, principally focused on the treatment of glioblastoma multiforme, melanoma, recurrent head and neck tumors, and liver metastases. To date, the clinical results showed a therapeutic advantage, associated to a higher quality of life and survival (e.g. Menendez et al. 2009; Kankaanranta et al. 2012; Kageji et al. 2014; Wang, Liu, Chou, Jiang 2018). The development of more selective boron compounds, new strategies and the use of accelerators based in hospitals will conceivably improve tumor response, reduce BNCT induced radiotoxicity and promote new clinical trials for those tumor targets that have already been explored and new ones (Schwint et al. 2019; Suzuki 2020). In this sense, BNCT translational studies in animal models are of outmost importance.

Our previous studies explored different carcinogenesis protocols based on the topical application of 0.5% of DMBA in mineral oil in the right cheek pouch of Syrian hamsters to evaluate different aspects of oral cancer therapy (Heber et al. 2010). The 12-week protocol, named ‘‘classical’’ protocol, is used for our short-term (one month) BNCT tumour control studies (e.g. Monti Hughes et al. 2017). However, this protocol has an important limitation: DMBA

cancerization induces initial severe oral mucositis and necrosis, affecting animal´s welfare and leading to tissue loss. The resulting short pouches are not useful for our BNCT studies because they cannot be everted for local neutron irradiation. Likewise, they would not be useful for other local treatments that require the pouch to be everted. Salley (1957) noted that the hamster cheek pouch exposed to DMBA goes through four distinct phases during the process of carcinogenesis, namely inflammation, degeneration, regeneration and hyperplasia. Evenson (1981) also reported that inflammation and necrosis was followed by healing and shrinkage of the pouch.

The aim of the present study was to optimize the classical cancerization protocol to avoid initial severe mucositis and pouch shortening, without affecting tumor development and the histological characteristics of tumor and precancerous tissue. We then performed BNCT studies employing hamsters submitted to this optimized protocol to study BNCT tumor response and associated mucositis, to evaluate if this optimization could affect BNCT tumor response and BNCT induced radiotoxicity. This optimization of the classical cancerization protocol would improve animal´s welfare and reduce the number of animals needed to be cancerized for our BNCT studies.

Materials and methods

This study was performed in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures, or with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and in accordance with local laws and regulations. All protocols were approved by the National Atomic Energy Commission Animal Care and Use Committee (CICUAL-CNEA, 04/27/2018, #02).

1. Optimization of the classical cancerization protocol

1.1 Experimental groups

The right cheek pouches of non-inbred young Syrian hamsters were submitted to what we term herein the classical carcinogenesis protocol, i.e. a topical application of 0.5% DMBA in mineral oil, twice a week, for 12 weeks (Garabalino et al. 2013). The classical protocol reduced the 3 weekly topical applications of the carcinogen in the standard hamster cheek pouch carcinogenesis protocol of Shklar, Eisenberg, Flynn (1979) to only 2 weekly applications. This modification was previously established to avoid a relatively high incidence of ascites (Heber et

al. 2010). It is known that numerous DMBA applications cause liver disorders such as enhanced oxidation of lipids and proteins coupled to compromised antioxidant defenses, contributing to animal decline (Letchoumy, Chandra Mohan, Kumaraguruparan, Hara, Nagini 2006; Molinari et al. 2011).

Animals were cancerized with two different cancerization protocols: <Group 1> The classical carcinogenesis protocol without DMBA interruptions during cancerization (24 applications); <Group 2> A carcinogenesis protocol with two DMBA interruptions corresponding to the 4th and 5th applications. These 2 skipped applications were completed at the end of the cancerization protocol (24 applications) (**Figure 1**).

1.2 Follow up

During cancerization and after the end of each cancerization protocol, clinical signs and body weight of the animals were monitored weekly. Mucositis due to the cancerization protocol was evaluated before each DMBA application (twice a week) upto mucositis resolution. Once mucositis resolved, and during the two months following the end of the cancerization protocol, the animals were followed once a week. Mucositis was analysed semi-quantitatively by visual inspection according to an adaptation of oral mucositis scales in humans and hamsters (Sonis et al. 2000; López-Castaño, Oñate-Sánchez, Roldán-Chicano, Cabrerizo-Merino 2005), i.e.: Grade 0 (G0): healthy appearance, no erosion, or vasodilation; G1 (slight): erythema and/or edema and/or vasodilation, no evidence of mucosal erosion; G2 (slight): severe erythema and/or edema, vasodilation and superficial erosion; G3 (moderate): severe erythema and/or edema, vasodilation and formation of ulcers <2 mm in diameter; G4 (severe): severe erythema and/or edema, vasodilation and formation of ulcers ≥2 mm and <4 mm in diameter, and/or areas of necrosis <4 mm in diameter; G5 (severe): formation of ulcers and/or areas of necrosis ≥4 mm in diameter. Grading was based on the most severe macroscopic feature.

Using this mucositis scale the following end-points were evaluated: (1) Time at which we observed the peak mean mucositis score; (2) Percentage (%) of animals with severe mucositis (Grade 4/5); (3) Number of days spent with severe mucositis (Grade 4/5). After mucositis resolution, we measured the length of the cancerized hamster cheek pouch, using as a reference the long axis of the pouch shelf used to locally irradiate the animals in our BNCT studies (**Figure 2**, zones 1+2+3). **Figure 2a** shows the ^{6}Li carbonate shielding device used to protect the body of

the hamster from the neutron flux while the cheek pouch bearing tumors is everted out of the enclosure onto a protruding shelf for irradiation during BNCT studies (Pozzi et al. 2009).

The pouch was considered <Long> if it covered zones 1+2+3 of the long axis of the pouch shelf (**Figure 2b**), <Medium> if it covered 2/3 of the long axis of the pouch shelf (zones 2+3, **Figure 2c**), and <Short> if it covered less than 1/3 of the long axis of the pouch shelf (zone 3, **Figure 2d**). Long pouches were considered optimum for irradiation, medium pouches were considered sub-optimum and short pouches were considered inadequate for our local irradiations.

Tumor development was evaluated considering those exophytic tumors that reached a volume of ≥1 mm^3 and ≥0.7 mm in height (González et al. 2017). Tumor volume was determined by external calliper measurement of the three largest orthogonal diameters (d) and calculated as d1 x d2 x d3 (e.g. Molinari et al. 2011). We assessed the percentage (%) of animals with tumors at 2 weeks, 4 weeks (one month) and 8 weeks (2 months) after the end of each cancerization protocol. Two months after finishing the cancerization protocol, we performed histological analyses of tumors and the precancerous tissue around tumors. The animals were euthanized and tissue samples of the cancerized and contralateral pouch (non-cancerized) were removed and fixed in 10% buffered formaline, paraffin embedded and sectioned at 5 μm. The sections were stained with haematoxylin-eosin and mounted.

2. BNCT studies

The animals were cancerized with the non-interrupted and the twice-interrupted cancerization protocol [Group 1 (11 animals) vs Group 2 (7 animals), respectively] and then exposed to BNCT mediated by BPA, "BPA-BNCT". The choice of the boron compound BPA is based on its clinical relevance (it is approved for use in humans and widely used in clinical trials) and has proved therapeutically effective in our translational studies (e.g. Kreimann et al. 2001; Pozzi et al. 2009). BPA was administered as a bolus intravenous (iv) injection at a dose of 15.5 mg ^{10}B/kg. Neutron irradiation was performed 3 h post administration of BPA. The animals were irradiated at the RA-3 thermal facility employing a lithium-6 carbonate shielding to protect the body of the animal while the cheek pouch is everted out of the enclosure onto a protruding shelf for exposure (**Figure 2a**). Total absorbed dose was prescribed to precancerous tissue and was 2.6 Gy. Dose calculations were performed considering 10 ppm as the boron concentration value in precancerous tissue (Kreimann et al. 2001; Monti Hughes et al. 2017).

BNCT induced mucositis was assessed at 7, 10, 14, 21 and 28 days after BNCT, considering the oral mucositis scale described in 1.2 in this section. We calculated the percentage (%) of animals with severe mucositis (Grade 4/5). Once a week during one month after BNCT, we evaluated tumor response in terms of tumor volume calculated as described in 1.2 (this section). The tumors were divided in different volume categories at the time of irradiation: small ($t < 10$ mm^3), medium (100 $mm^3 > t \geq 10$ mm^3), large ($t \geq 100$ mm^3). Then, for each category, we assessed: % of tumors with complete response (CR: disappearance of the tumor on visual inspection); % of tumors with partial response (PR: reduction in pre-treatment tumor volume); % of tumors with no response (NR); % of tumors with overall response (OR) = partial response (PR) + complete response (CR).

3. Statistical analysis

Statistical analysis was carried out with the R statistical program (R Development Core Team 2019). The percentage of animals with severe mucositis and of animals with long pouches were analyzed with Generalized Linear Models (GLM, using the package stats; R Core Team & contributors worldwide 2019) with the Binomial error structure, a logit-link function and the Laplace approximation method (Bolker et al. 2009; Zuur, Ieno, Walker, Saveliev, Smith 2009; Crawley 2012). To assess the performance of the models we used the "Kappa (k)" index (Cohen 1960), considering the labels of the relative strength of agreement designed by Landis and Koch (1977). The Presence-absence package (Freeman 2012) was used to estimate the sensitivity, the specificity and the k index. Analyses of tumor development, CR, PR, NR and OR parameters were performed using log-linear models (using the package stats; R Core Team & contributors worldwide 2019). The significance of GLM and log-linear models were evaluated using Chi-squared Analysis of Deviance (Crawley 2012). The differences in mean peak mucositis were evaluated by Student's t test. Statistical significance was set at $p=0.05$.

Results

1. Optimization of the classical cancerization protocol

In the group of cancerized animals without interruptions (Group 1), 12% of the animals (2/17) had to be euthanized one week after starting the cancerization protocol. These animals exhibited clinical decline and grade 5 mucositis (severe) in the cancerized pouch. In this group, 71% of the animals exhibited severe mucositis (Grade 4/5), appearing at the time of the 5th DMBA

application and lasting upto the time of the 10th application (**Table 1, Figure 3**). The mean peak mucositis score was 2.7±1.9 at the time of the 7th DMBA application (**Table 1**). At the end of the protocol, only 53% of the animals exhibited long pouches (**Table 1, Figure 3**).

Based on these results, we decided to interrupt the cancerization protocol at the time of the 4th and the 5th DMBA applications. We skipped the 4th and 5th applications based on the working hypothesis that in this way it would be possible to reduce the incidence of severe mucositis. Only 1 of the 18 animals had to be euthanized one week after the beginning of the cancerization protocol due to clinical decline and mucositis severity. Only 17% of the animals of this group exhibited severe mucositis, being significantly lower than for Group 1 (17% vs 71%, $p<0.001$, sensitivity = 0.8±0.1, specificity = 0.8±0.1, kappa = 0.5±0.1 [moderate strength of agreement]) (**Table 1**). The 2 remaining animals that exhibited severe mucositis did so for a brief span (at the time of the 8th application). The majority of the animals exhibited only Grade 1 / Grade 2 mucositis (**Table 1, Figure 3)**. The mean peak mucositis score was reached at the time of the 8th application, being significantly lower than for Group 1 (1.4±1.1 vs 2.7±1.9, $p=0.0226$). 100% of the hamsters in this group exhibited long pouches after finishing the cancerization protocol. This value was statistically higher than for Group 1 (53%, $p<0.001$, sensitivity = 0.7±0.1, specificity = 1±0, kappa = 0.5±0.1 [moderate strength of agreement]; **Table 1, Figure 3**).

In both groups, after severe mucositis resolution, the animals fluctuated between Grades 0, 1 and 2 mucositis and occasionally reached Grade 3.

Regarding tumor development, we observed that these two interruptions in Group 2 did not affect tumor development versus Group 1 ($p=0.4633$; **Table 2**).

Finally, the histological analysis showed that the interruptions performed did not affect tumor or precancerous tissue characteristics. **Figure 4** shows a representative example of a cancerized hamster cheek pouch bearing tumors with its corresponding histological image for Groups 1 and 2. Both groups exhibited semi differentiated infiltrating squamous cell carcinomas with a scarce stromal reaction, and cells with moderated atypia, bizarre nuclei and mitosis.

2. BNCT studies

Table 3 shows the therapeutic effect of BNCT on tumors in Group 1 and 2. In both groups, BNCT mediated by BPA induced a high tumor overall response (OR), without evidence of statistical differences (86% for Group 1 and 83% for Group 2; $p=0.7462$) (**Table 3**). The parameters CR, PR, and NR did not show any statistical differences between both groups

(p=0.873; **Table 3**). Regarding radiotoxicity, we observed a high, statistically similar, incidence of severe mucositis induced by BNCT for Groups 1 and 2 (73% vs 86% respectively, p=0.5087).

Discussion

For HNSCC, radiotherapy with or without concurrent chemotherapy has been established as primary treatment, however, there is still a 50% of patients that suffer loco(regional) failure (Elbers et al. 2019). Surgery is also an option, but is sometimes mutilating, affecting patient quality of life. In the last years, the study of new targeted therapies showed an improvement in survival and a reduction in therapy toxicity (Li et al. 2018, Monti Hughes et al. 2019). BNCT is a targeted therapy that allows for higher doses to tumor while sparing normal tissue. Our group proposed the hamster cheek pouch oral cancer model to study BNCT for head and neck cancer, preceding the first clinical trial of BNCT for head and neck cancer (Kreimann et al. 2001, Kato et al. 2004).

Our studies seek to optimize BNCT for head and neck cancer, evaluating new boron compounds and strategies in the hamster cheek pouch oral cancer model (e.g. Trivillin et al. 2006, Molinari et al. 2011, Heber et al. 2014, Garabalino et al. 2019). Research on live animals is necessary to understand how cancers develop and spread throughout the body, to study cancer diagnosis and improve cancer treatments (Workman et al. 2010). The welfare of animals in cancer research is defended from an ethical point of view and also considered consistent with good science (Osborne, Payne, Newman 2009). In this sense, all experiments should incorporate the 3Rs and be implemented throughout the lifetime of the study: replacement (of animals with alternative methods), reduction (in the numbers of animals used) and refinement (of methods to minimise animal suffering) (Russell & Burch 1959). Focusing on animal welfare, researchers must follow published recommendations for study design, statistics and pilot studies.

The model of chemical cancerization in the hamster cheek pouch is the most widely accepted experimental model for oral cancer, in which the solution of the carcinogen is spread over the whole mucosa, similarly to tobacco and alcohol, and induces premalignant and malignant changes that represent very closely the spontaneous human oral mucosa lesions (Monti Hughes et al. 2015b). In this sense, animals cannot be replaced with alternative methods, but the studies should follow the other two principles: refinement and reduction. We demonstrated that the classical cancerization protocol induced early severe mucositis. Oral mucositis causes pain, mal nutrition and low quality of life. Within this context, in this study we optimized the classical

cancerization protocol (DMBA application twice a week, during 12 weeks), interrupting the cancerization protocol by skipping 2 DMBA applications at the beginning of the protocol and completing them at the end. In this way we were able to reduce severe mucositis, avoid pouch shortening and improve the clinical status of the animals during carcinogenesis, without affecting tumor development after the end of the protocol. We also demonstrated that BNCT induced a similar effect, in terms of tumor control response and incidence of severe mucositis, in animals cancerized with the twice-interrupted protocol and in animals cancerized with the non-interrupted classical protocol. The fact that interruptions of the DMBA protocol resulted in a lower percentage of animals with severe mucositis without affecting tumor development or tumor and precancerous tissue histological characteristics, is consistent with the principle of refinement, which aims at minimising animal suffering. Besides, these two interruptions resulted in 100% of long pouches optimum for irradiation. This outcome allows us to reduce the number of animals that have to be cancerized to perform our BNCT studies. This aspect is consistent with the principle of reduction.

Salley (1957) reported that histological changes were apparent in pouches treated with only one application of DMBA. These histological changes involved inflammation with the presence of inflammatory cells in the epithelium, the submucosal connective tissue and the striated muscle layers. The same findings were noted in the pouches which had received 2 treatments. When the pouch was subjected to 3 treatments of the carcinogen, the inflammatory reaction appeared to have subsided in intensity, especially in the submucosal connective tissue and the muscular layer. There was still some inflammation present in the epithelium, but the most prominent feature in this tissue was degeneration and necrosis. Also Mognetti et al. (2006) described Salley's findings in this model, explaining that during the first 2 weeks there was an inflammatory phase with necrosis and sloughing of the distal part of the pouch, followed by healing and shrinkage. These results are similar to our observations in terms of mucositis development. We observed that, employing the classical protocol without interruptions, animals with severe mucositis appeared mainly at the time of the 5th application, and with less frequency at the time of the second application. Based on the previous cited findings and our results, we decided to interrupt the 4th and 5th DMBA applications, expecting to reduce this inflammatory process and ensuing necrosis and pouch shortening. Our results showed that only a small number of animals exhibited severe mucositis yielding 100% of long pouches, optimum for our local irradiation studies.

Importantly, we observed that skipping the 4^{th} and 5^{th} DMBA applications during the carcinogenesis protocol did not affect tumor development after the end of the cancerization

protocol. Odukoya & Shklar (1982), attempting to apply the two-stage concept of carcinogenesis to the hamster buccal pouch model system, reported a procedure to study initiation and promotion of DMBA. They studied three different cancerization protocols, one of which had a 6 week-interruption. In this interrupted protocol, tumor development was not impaired either.

DMBA-induced squamous cell carcinomas in hamsters have similar morphological, histological and genetic features to human oral SCC (Vairaktaris et al. 2008). Some studies focused on the evaluation of new therapeutic approaches and chemopreventive compounds, meanwhile others concentrated on the evaluation of the expression of biomarkers related to sequential carcinogenesis (Yapijakis, Kalogera, Papakosta, Vassiliou 2019). Our study focused on the optimization of the hamster cheek pouch oral cancer model to study therapeutic alternatives. Particularly, we studied BNCT for the treatment of oral cancer as our group is focused on this targeted therapy since 2001 (Kreimann et al. 2001). As to biomarkers, several studies concentrated on this topic. A comparative study between humans and hamsters showed aberrant expression of multiple molecules in key signalling pathways in both human and hamster oral SCC induced by 14 weeks of DMBA application (Nagini et al. 2009). Also Hsue et al. (2008a,b) demonstrated the expression of inhibitors of an apoptosis family protein and p53 accumulation in 3 different carcinogenesis protocols, with different lengths of cancerization, i.e. 3-week, 7-week, 14-week DMBA applications. In our study, DMBA interruptions were performed in the 4^{th} and 5^{th} DMBA application, i.e. at the end of the 2^{nd} week and starting the 3^{rd} week of cancerization. In this sense, these two interruptions should not affect the induced molecular oncological pathways, as Hsue et al. (2008a,b) demonstrated that a 3-week DMBA cancerization protocol also induces the expression of key molecules for SCC development in the hamster cheek pouch. Besides, as our results showed no differences in tumor development and in tumor histological characteristics between both cancerization protocols, our results support the results of Hsue et al.. To complete the comparison between the non-interrupted and interrupted protocols, for our BNCT studies it was very important to evaluate if DMBA interruptions affected the therapeutic effect of BNCT on tumors and induced mucositis in precancerous tissue. So, we performed BNCT studies in both groups and demonstrated that this optimization of the cancerization protocol did not affect the outcome in terms of tumor control or radiotoxicity, rendering it suitable to pursue our BNCT studies.

Conclusion

In this study, we proposed an optimized cancerization protocol in the hamster cheek pouch for the study of oral cancer therapy, suitable in particular for BNCT studies, which improves animal´s welfare and reduces the number of animals needed to be cancerized for each study.

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Figure Legends

Figure 1. Schematic representation of cancerization protocols: <Group 1> Cancerization protocol without DMBA interruptions during cancerization; <Group 2> Cancerization protocol with two DMBA interruptions: corresponding to the 4th and 5th applications. These 2 interruptions were completed at the end of the cancerization protocol.

Figure 2. Representative examples of hamster cheek pouch length: (a) Lithium-6 carbonate shielding device to protect the body of the animal while the cheek pouch is everted out of the enclosure onto a protruding shelf for irradiation. The shielding device is shown without its lid. (b) Long: the pouch covers zones "1+2+3"; (c) Medium: the pouch covers zones "2+3"; (d) Short: the pouch only covers zone "3". Short pouches were not considered useful for our local irradiations.

Figure 3. Mucositis development, resolution and wound healing in Group 1 (non-interrupted) and Group 2 (twice-interrupted) cancerization protocols. Group 1: a representative example of a hamster exhibiting Grade 5 (severe mucositis with necrosis -N-) and a short pouch at the end of the cancerization protocol. Group 2: a representative example of a hamster exhibiting Grade 2 mucositis and a long pouch at the end of the cancerization protocol. Long pouches were considered optimum for irradiation whereas short pouches were considered inadequate for our local irradiations.

Figure 4. Representative example of a cancerized hamster cheek pouch bearing tumors with its corresponding histological image for Group 1 (No interruptions; Panels a, b, c) and Group 2 (With two interruptions; Panels d, e, f). Both groups exhibited semi differentiated infiltrating squamous cell carcinomas with scarce stromal reaction (b and e, respectively) and cells with moderate atypia, bizarre nuclei and mitosis (c and f, respectively).

Table 1. Number of euthanized animals, percentage (%) of animals with long pouch, mean peak mucositis and percentage (%) of animals with severe mucositis for Groups 1 (non-interrupted) and 2 (twice-interrupted). n= number of animals evaluated for each parameter.

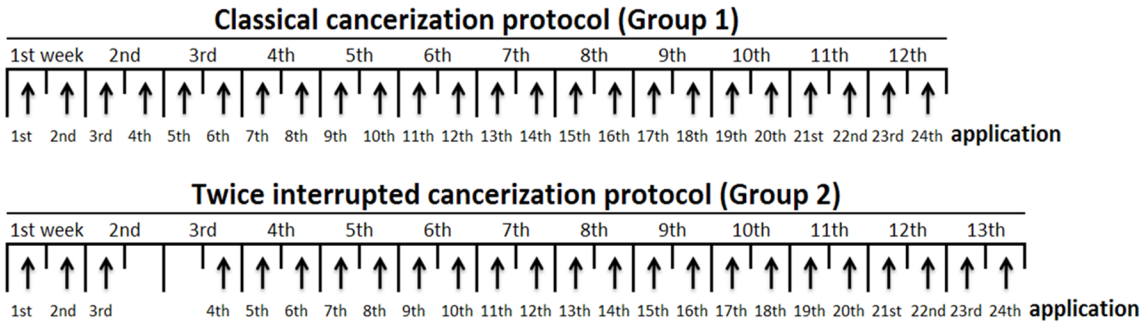
	Number of euthanized animals	**% of animals with long pouch**	**Mean Peak Mucositis**	**% of animals with severe mucositis**
Group 1	2 (n=17)	53 (n=15)	2.7±1.9	71 (n=17)
Group 2	1 (n=18)	100 (n=17)	1.4±1.1	17 (n=18)

Table 2. Percentage (%) of animals and number of animals with tumors at 2 weeks, 1 month and 2 months after the end of the cancerization protocol for Group 1 (non-interrupted) and Group 2 (twice-interrupted). n= number of animals.

	2 weeks		**1 month**		**2 months**	
	% animals with tumors	n	% animals with tumors	n	% animals with tumors	n
Group 1	78%	7 of 9	100%	9 of 9	100%	8 of 8
Group 2	90%	9 of 10	100%	10 of 10	100%	9 of 9

Table 3. BNCT studies for each cancerization protocol (Groups 1 and 2), 1 month after treatment: Percentage (%) of tumors (Large, Medium and Small at the time of irradiation) with No Response (NR), Partial Remission (PR), Complete Remission (CR) and Overall Response (PR+CR).

	Tumors (t)	No response (NR)	Partial remission (PR)	Complete remission (CR)	Overall tumor response (PR+CR)
Group 1 (non-interrupted)	Total (n=35)	14.3%	25.7%	60.00%	**85.7%**
	Large: t ≥ 100mm^3(n=2)	0.0%	0.0%	100.0%	100.0%
	Medium: 100 mm^3>t ≥ 10mm^3(n=9)	22.2%	55.6%	22.2%	77.8%
	Small: t< 10mm^3 (n= 24)	12.5%	16.7%	70.8%	87.5%
Group 2 (twice-interrupted)	Total (n=29)	17.2%	20.7%	62.1%	**82.8%**
	Large: t ≥ 100mm^3(n=4)	25.0%	25.0%	50.0%	75.0%
	Medium: 100 mm^3>t ≥ 10mm^3(n=8)	12.5%	62.5%	25.0%	87.5%
	Small: t< 10mm^3 (n= 17)	17.6%	0.0%	82.4%	82.4%



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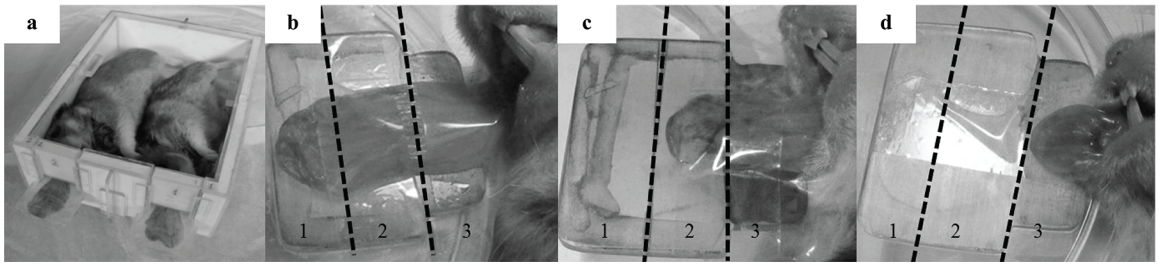

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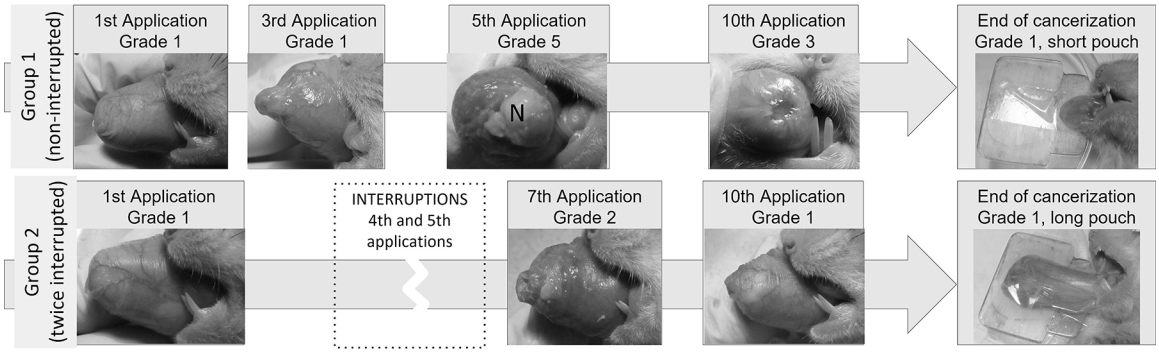

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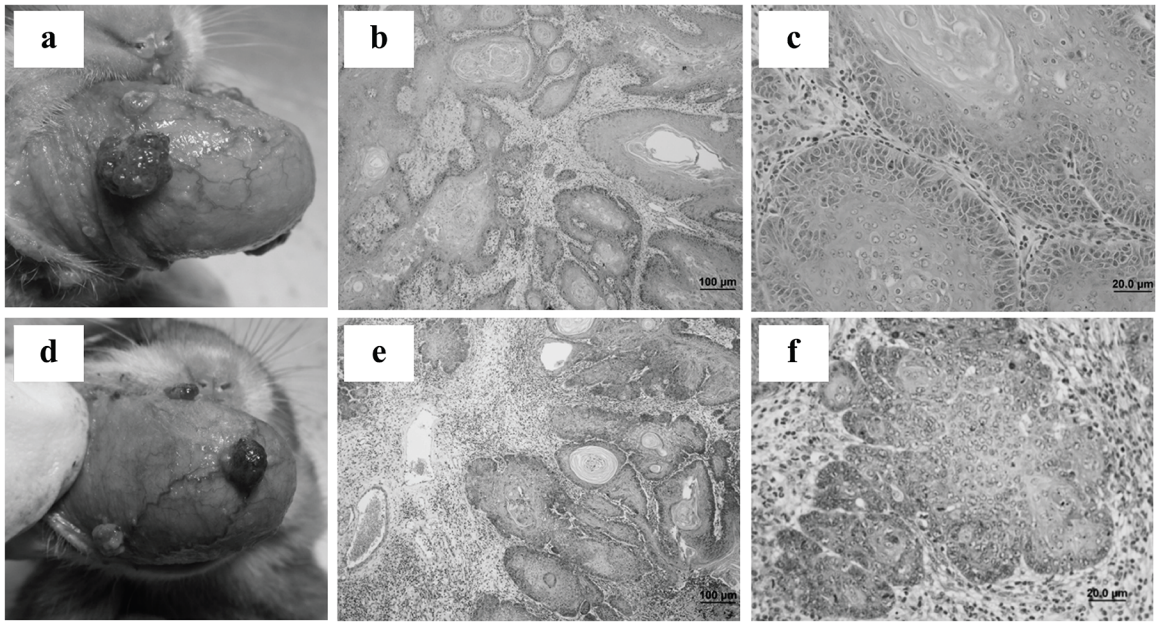

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