Biological effects of brachytherapy using a 32P-patch on the skin of Sencar mice

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Abstract. In recent years, specially designed patches containing beta emitters have been developed for contact brachytherapy of skin lesions. The aim of the present work is to evaluate the biological effects of the 32P-patch on the skin of Sencar mice as a result of a brachytherapy treatment. For this purpose, a 32P-patch was prepared with Chromic 32P-phosphate and silicone and the classical model of two-stage skin carcinogenesis was reproduced in Sencar mice. Animals were divided in two main groups in order to perform the contact brachytherapy treatment using schemes of single (SD40 and SD60) and fractionated (FD40 and FD60) doses, with their respective control groups (CSD and CFD). Additionally, a control group without carcinogenic treatment was included in order to apply the 32P-patch in normal skin. The endpoint to evaluate treatment effects was tumor size after a follow-up period of 44 days and finally, animals were sacrificed in order to get samples of all tumors for histological analysis. Additionally, PCNA staining was evaluated in all groups and the biologically effective dose (BED) of each scheme was calculated taking into account the linear-quadratic model. Erythema, dermatitis and skin ulceration developed in almost all treated animals, but they gradually healed with regeneration of tissue during the follow-up period. Radiation effects on the skin of SD40, SD60, FD40 and FD60 showed a significant reduction of the tumor size with regard to controls, independently of the scheme and the radiation dose considered. PCNA staining scores of groups in the single dose scheme resulted higher for control than for treated tumors, and the same pattern was observed for groups of the fractionated dose scheme. This radioactive 32P-silicone-patch which is easy to prepare and use in the treatment of skin diseases and seems promising as a radioactive device for clinical use.

KEYWORDS: 32P, brachytherapy, skin cancer, patch, Sencar mice.

1. Introduction

Treatment modalities for skin diseases such as melanoma, non-melanoma skin cancer and keloids are surgical excision, radiotherapy and chemotherapy [1-3]. Each therapeutic mode has its own advantages and disadvantages, but brachytherapy has been reported as a promising effective alternative. In recent years, specially designed patches containing beta emitters such as 90Y, 188Re and 166Ho have been developed for contact brachytherapy of skin lesions [4-8]. The experience in treating ophthalmologic diseases was the basis for the design of such brachytherapy treatments, employing both gamma and beta emitters. These kinds of devices fulfil the advantages of localized brachytherapy treatments and were designed according to the shape of the lesions with variable activity of the radionuclide with regard to the different types of treatment schemes applied. Since the availability of these beta emitters, their cost and their physical characteristics are of importance when considering the possibility for their employment for treatment, we designed a 32P-patch, as this radioisotope is commercially available in Southamerica. In previous works we evaluated the 32P-patch with regard to
its radiopharmaceutical characteristics demonstrating the absence of radioactivity leakage in vitro and in vivo as well as the homogeneous distribution of the radionuclide in the patch [9-10]. The aim of the present work is to evaluate the biological effects on the skin of Sencar mice as a result of a brachytherapy treatment.

2. Materials and methods

2.1 32P-patch production and control

For the production of patches, 10 mCi of 32P were purchased as Phosphoric-32P-acid (CNEA, Buenos Aires, Argentina). The patches were prepared as previously described [9-10]. Briefly, we used Chromic 32P-phosphate (30-70 nm) and silicone (Silastic®J-White 80, Dow Corning, The Dow Chemical Company and Corning, Inc., USA). The Chromic 32P-phosphate was washed with isopropilic alcohol (Anhedra, Argentina), centrifuged to 2000 rpm during 10 minutes and finally dried at 80ºC in order to obtain a fine powder. Afterwards, the Chromic 32P-phosphate powder was mixed with the silicone and dried at room temperature during 4 hours.

The patches were designed taken into account the size and the shape of the tumors and in the case of control animals we used these same patches in order to mimic treatments. For experimental purposes the patches were not shielded.

The activity concentration of patches was measured as previously described [9-10]. Briefly, a sample of the patches was dissolved with hyamine hydroxide (MP Biomedicals, LLC, USA) at room temperature overnight and an aliquot was added to a vial containing 3 mL of a complete phase combining system for liquid scintillation counting (PCS® Amersham Biosciences, USA). The activity measurement was performed in a liquid scintillation counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia Wallac OY, Finland) according to the 32P protocol, with a relative error <1%. Results were expressed as kBq/cm² (or µCi/cm²) taking into account the weight of the sample and the density of the patches.

2.2 Animals

All animal experiments were performed in accordance with the “Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996”. We used female mice of Sencar strain (CNEA, Argentina) 7-9 weeks old. The animals were fed ad libitum with balanced standard food and maintained in stainless steel cages with cycles of 12/12 hours light/darkness and controlled room temperature.

The classical model of mouse two-stage skin carcinogenesis [11-13] was reproduced in order to evaluate biological effects of radiation on skin. Briefly, the backs of the mice were shaven with surgical clippers at least 3 days prior to treatment and only mice in the resting phase of the hair cycle were used. Mice were initiated by a single topical application of 20 nmol of DMBA (7,12-dimethylbenz[a]anthracene, Sigma Chemical Company, St. Louis, MO, USA) in 0.2 ml of acetone. Afterwards, they were topically treated twice a week with TPA (12-O-tetradecanoylphorbol-13-acetate, Sigma Chemical Company, St. Louis, MO, USA) 3.25 nmol in 0.2 ml of acetone, starting ten days after initiation and over a period of 20 weeks. Control animals were submitted to the same treatment described but we used saline solution instead of DMBA and TPA.

2.3 Radiation treatments

We performed two contact brachytherapy treatments employing a single and a fractionated dose scheme. For the single dose scheme we administered total physical doses of 40 and 60 Gy. For the fractionated dose scheme we administered two doses of 40 and 60 Gy each, with an interval of a week between them.

2.4 Biological effects of radiation on skin

We used 56 animals that were randomized in two main groups: controls without carcinogenic treatment (C, n=10); and treated with carcinogen (n=46). Animals treated with carcinogen were
afterwards divided in the following groups: controls without brachytherapy treatment (CWB, n=7); treated group with $^{32}$P-patch in a single dose scheme of 40 Gy (SD40, n=10); treated group with $^{32}$P-patch in a single dose scheme of 60 Gy (SD60, n=9); controls treated with a decayed $^{32}$P-patch mimicking the single dose scheme (CSD, n=6); treated group with $^{32}$P-patch in a fractionated dose scheme of two doses of 40 Gy (FD40, n=9); treated group with $^{32}$P-patch in a fractionated dose scheme of two doses of 60 Gy (FD60, n=10); and controls treated with a decayed $^{32}$P-patch mimicking the fractionated dose scheme (CFD, n=4). Initial tumor sizes were recorded for all animals. Afterwards, each patch was applied into direct contact with lesions or the normal skin selected to be treated, and further firmly affixed with a hypoallergenic adhesive tape to prevent displacement. The time of exposition for each physical dose was determined according to dosimetric calculations described below. After 10 days of the end of the treatments, TPA was again administrated to all animals with the same frequency during the follow-up period except for C group which received saline solution, instead. The follow-up period was of 44 days for all groups during which tumor size was recorded as the endpoint. Finally, animals were sacrificed in order to get samples of all tumors for histological analysis and PCNA staining.

2.5 Dosimetric calculations

Equation 1 describes the estimation of the average absorbed dose in a tumor of thickness equivalent to the maximum range of $^{32}$P in the absorber considered. This equation derives from the MIRD DOSE scheme (14).

$$\bar{D} = \frac{2.13}{m} \sum_{i} n_{i} \bar{E}_{i} \Phi \left( \text{cGy} \right)$$  \hspace{1cm} \text{Equation 1}

Where:

a) $2.13 = k$ is a constant to convert units to express the result in cGy.

$$k = 1.602 \times 10^{-6} \text{ erg MeV}^{-1} \times 3.7 \times 10^{4} \text{ dis s}^{-1} \mu\text{Ci}^{-1} \times 10^{-2} \text{ cGy g}^{-1} \text{ erg}$$

b) $\frac{\bar{A}}{m}$ represents the accumulated activity per unit mass, which can be calculated as described in Equation 2.

$$\frac{\bar{A}}{m} = As \left( 1 - e^{-\frac{\ln 2}{\frac{R_{\beta}}{2}}} \right)$$  \hspace{1cm} \text{Equation 2}

Where: 
- As: activity per unit of surface expressed in ($\mu$Ci / cm$^2$).
- $R_{\beta}$: maximum range in tissue expressed in cm (for $^{32}$P is 0.75 cm).
- $\delta$: density of tissue simulated as water 1g/cm$^3$.

c) $\sum_{i} n_{i} \bar{E}_{i} = \Phi$ represents the probability of the transition and its energy associated according to the disintegration mechanism of the radionuclide. In the case of $^{32}$P, $\bar{E}_{i}$ is 0.7 MeV.

d) $\Phi = 0.5$ since the source is plane and only one face is in contact with skin.
The biologically effective dose (BED) of each scheme was calculated taken into account the linear-quadratic model \((\text{equation 3})\) as extensively explained elsewhere [15].

\[
\text{BED} = D \left[ 1 + \frac{D g}{\alpha / \beta} \right]
\]

\text{Equation 3}

Where

\(D\) = dose rate x time

\(\alpha / \beta\) = tissue radiosensitivity considered as 10 Gy [2].

\(g\) = incomplete repair factor calculated as \text{equation 4}

\[
g = 2 \left[ \frac{\mu t - 1 + e^{-\mu t}}{\left(\mu t\right)^2} \right]
\]

\text{Equation 4}

Where

\(t\) = time

\(\mu\) = subletal damage rate of repair calculated as \text{equation 5}

\[
\mu = \frac{\ln 2}{t_{1/2\text{rep}}}
\]

\text{Equation 5}

Where \(t_{1/2\text{rep}}\) = subletal damage half repair time considered as 1 hour [2].

2.6 Histological studies

All samples were processed for conventional histological examination by formalin fixation and paraffin embedding. Sections were stained with haematoxylin and eosin. Photomicrographs of haematoxylin and eosin sections of all samples were taken at 10X and 400X magnification using a Canon PowerShot G5 camera (Japan).

2.7 PCNA staining

Paraffin sections of control and treated tumors after deparaffinization were placed in citrate buffer (10mM, pH 6.0) and heated in a microwave oven twice for 2 minutes at boiling temperature for antigen retrieval. Endogenous peroxidase activity was blocked with 3% \(\text{H}_2\text{O}_2\) in distilled water. Specimens were then incubated overnight in a humidified chamber at 4°C with primary mouse anti-PCNA (1: 100, DakoCytomation, Denmark) antibodies, as stated. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse and visualized by 3,3-diaminobenzidine staining (Sigma Chemical Co., MO, USA). Finally, the specimens were counter-stained by immersion in haematoxylin. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Germany).

The immunostaining assessment was performed by consensus of 2 independent observers. An overall examination of staining was carried out at 10X magnification, and representative area of the specimen was then view at 100X magnification. A percentage score based on the number of stained cells assigned as: 0 (undetectable), 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80%) and 5 (81-100%). Determinations were made over at least 25 fields examined.

2.8 Statistical analysis

Tumor growth at the end of the follow-up period as well as PCNA staining of control and treated tumors were compare using the ANOVA test or the Kruskall-Wallis test with a significance level of \(p<0.05\), followed by the Student-Newmans-Keuls test or by the Dunn test, respectively in order to evaluate differences among groups as the \textit{a posteriori} tests [16]. The results are shown as means ± standard deviation.
3. Results
The results of dosimetric calculations as well as a brief summary of the treatment protocols is shown in table 1. In order to determine the time of exposition of the $^{32}$P-patches to each group according to the physical dose ($D$) selected in the protocol, the accumulated activity per unit mass was calculated from equation 1. Then, the activity per surface unit of the $^{32}$P-patches measured by liquid counting scintillation was applied in equation 2 to finally obtain the time of exposition. Additionally, table 1 also shows the calculated $g$ factor as described in equation 4 and the BED calculated according to the linear-quadratic model as described in equation 3.

Table 1: Dosimetric calculations results.

<table>
<thead>
<tr>
<th>Treatment scheme</th>
<th>Sessions</th>
<th>Interval between sessions</th>
<th>$^{32}$P-patch ($\mu$Ci/cm$^2$)</th>
<th>t (h)</th>
<th>g</th>
<th>BED per session (Gy)</th>
<th>Total BED per treatment (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{D} = 40$ Gy</td>
<td>1</td>
<td></td>
<td>263</td>
<td>15.5</td>
<td>0.169</td>
<td>67.0</td>
<td>67.0</td>
</tr>
<tr>
<td>$\bar{D} = 60$ Gy</td>
<td>1</td>
<td></td>
<td>263</td>
<td>23.0</td>
<td>0.118</td>
<td>102.5</td>
<td>102.5</td>
</tr>
<tr>
<td>Fractionated dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{D} = 40$ Gy</td>
<td>2</td>
<td>1 week</td>
<td>#1</td>
<td>341</td>
<td>12.0</td>
<td>0.212</td>
<td>73.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#2</td>
<td>274</td>
<td>15.0</td>
<td>0.174</td>
<td>67.8</td>
</tr>
<tr>
<td>$\bar{D} = 60$ Gy</td>
<td>2</td>
<td>1 week</td>
<td>#1</td>
<td>341</td>
<td>18.0</td>
<td>0.147</td>
<td>112.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#2</td>
<td>274</td>
<td>22.5</td>
<td>0.120</td>
<td>103.2</td>
</tr>
</tbody>
</table>

The two-stage carcinogenesis protocol produced both papillomas and keratoacanthomas as described elsewhere [15-17], which were confirmed with histological analysis (figure 1, panel A and B). After completion of therapy with the $^{32}$P-patch, erythema, dermatitis and skin ulceration developed in animals of group C and in animals which tumors had macroscopically dissapeared of the other groups, but they gradually healed with regeneration of tissue during the follow-up period. Histological findings of the skin of animals in group C, showed the hypotrophy of the skin as a consequence of radiation, with no alteration of epidermis and dermis (figure 1 panel C). The lost of hair follicles shows the limits of the irradiated zone of the skin.

Radiation effects on the skin of the other groups showed a significant reduction of the tumor size, expressed as diameter in milimeters, of treated tumors with regard to controls, independently of the scheme and the radiation dose considered. Figure 2 (panel A) shows the results for tumor size of groups in the single dose scheme, with significant tumor size reduction (p<0.05) in SD40 and SD60 with regard to control groups CWB, CSD. No significant differences in tumor size were found between SD40 and SD60 or between control groups. Brachytherapy with the $^{32}$P-patch resulted in the macroscopic disappearance of 3 of the 10 treated tumors with 40 Gy and of 5 of the 9 treated tumors with 60 Gy. Histological analysis showed 5 total remissions (1 from SD40 and 4 from SD60) and 3 partial remissions (2 from the SD40 and 1 from the SD60) of the disappeared tumors. Animals in the fractionated dose scheme groups also presented a significant reduction in tumor diameter after patch application in comparison with controls. Figure 2 (panel B) shows that tumor size of FD40 and FD60 significantly differed from control groups (p<0.05) but no differences were found within treated groups and neither within controls. In this case, brachytherapy treatment resulted in the macroscopic disappearance of 7 of the 9 treated tumors with a total dose of 40 Gy and of 8 of the 10 treated tumors with a total dose of 60 Gy. Histological analysis showed 9 total remissions (4 from the FD40 and 5 from the FD60) and 6 partial remissions (3 from the FD40 and 3 from the FD60) of the disappeared tumors.

PCNA staining scores of groups in the single dose scheme resulted higher for control than for treated tumors, showing significant differences for SD40 and SD60 with regard to CSD and CWB. (p<0.01, figure 3, panel A). The same pattern was observed for groups of the fractionated dose scheme, with significant differences for FD40 and FD60 with regard to CFD and CWB (p<0.01, figure 3, panel B). On the other hand, we did not found differences in PCNA scores between control groups neither
between treated groups independently of the therapeutic scheme employed or the physical doses assayed.

**Figure 1:** Hematoxilin-eosin staining photographs. Panel A: control papilloma. Panel B: control keratoacanthoma. Panel C: normal skin irradiated with $^{32}$P-patch.

![Figure 1](image)

**Figure 2:** Tumor size (diameter in mm) evolution after $^{32}$P-patch application. Panel A shows the results for groups in the single dose scheme. Panel B shows the results for groups in the fractionated dose scheme.

![Figure 2](image)

**Figure 3:** PCNA scores of tumors. Panel A shows the results for groups in the single dose scheme. Panel B shows the results for groups in the fractionated dose scheme.

![Figure 3](image)

**4. Discussion**

Brachytherapy has been reported as a valid alternative treatment modality for these diseases, in the last years [2, 3, 17]. In this work, we used $^{32}$P which emits $\beta$ radiation ($E_{\text{max}} = 1.7$ MeV) with a half life of 14.3 days, a maximum range in tissue of 7.5 mm and it is produced in nuclear reactor. This beta
emitter, extensively available in our country and Southamerica, has similar characteristics to those used by other groups with the additional advantages of its long half life, low cost and the absence of gamma radiation.

In order to evaluate the biological effects of the $^{32}$P-patch application on the skin of Sencar mice, we selected two therapeutic schemes employing physical doses of 40 and 60 Gy given in a single dose (single dose scheme) or two equal doses of 40 or 60 Gy with an interval between them of a week (fractionated dose scheme). Doses were calculated with a formula derived from MIRD DOSE scheme where the physical calculated dose is the average absorbed dose in a tumor of thickness equivalent to the maximum range of $^{32}$P in water and taken into account that the radiation source is plane and it is in contact with the skin. All treatments resulted in a low dose rate (LDR) treatment that for comparative purposes were expressed in terms of BED, calculated according to the linear-quadratic model. In this way, it is interesting to note that BED values are higher than physical dose values and on the other hand, fractionated scheme BEDs are not equal but higher to the arithmetical sum of the BEDs of the single dose scheme. All these differences may be explained by differences in the g factor of the BED formula, which takes into account tissue reparation as a consequence of the time elapsed during the treatments. In this case, repopulation was a factor not taken into account since the overall time of all treatments was less than a week or equivalent to a week [2, 15]. According to these calculations, radiation dose from $^{32}$P-patch application was higher for both fractionated scheme groups than for single dose groups and biological effects on skin related to these doses were observed as explained below.

Sencar mice that were not submitted to the carcinogenic protocol but received radiation from the $^{32}$P-patch application on skin, developed erythema, dermatitis and ulceration of skin which healed during the follow-up period. These observations correlated with the hypotrophy of the skin and the lost of hair follicles that appeared at the histological level. On the other hand, mice that developed papillomas and keratoacanthomas as a consequence of the carcinogenic protocol and treated with the $^{32}$P-patch showed tumor growth arrest as the principal biological effect independently of the therapeutic scheme assayed. Nevertheless, the higher the total dose, the higher the number of remissions observed. Indeed, the number of total and partial remissions as well as tumor growth control in terms of tumor size at the end of the follow-up period, was higher for the fractionated dose scheme and these results were confirmed by the histopathology and PCNA staining.

5. Conclusion

Our results show that the $^{32}$P-patch designed as a contact brachytherapy device is easy to prepare and use in the treatment of skin diseases. Biological effects of radiation from $^{32}$P-patch in Sencar mice skin showed promissory results in order to consider it as a valid therapeutic modality for clinical use in the treatment of skin diseases. Future perspectives will be focused in assaying different therapeutic schemes including other radiation doses in order to improve cure rates, as well as its use in other types of skin diseases.

6. References