LIGHT MICROSCOPIC EVALUATION
OF THE NUCLEOLAR ORGANISER REGIONS
OF SKIN CELLS OF THE DOGS SUBJECTED
TO THERAPEUTIC ULTRASOUNDS

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Abstract

A possible use of some argyrophilic nucleolar organiser region (AgNOR) parameters as a biological counter to predict the biological behaviour of basal epidermal cells and dermal fibroblasts in dogs subjected to therapeutic ultrasound (US) exposure was determined. All the dogs, except the control dogs, had US treatment at an intensity of 0.5 W/cm² (SATA), frequency of 1.0 MHz, pulsed 2 ms on and 8 ms off for 5 min daily for 10 d. Full thickness skin samples were taken on days 4, 18, 32, 46, and 74 following cessation of 10 d US application and right after the 10th d of US exposure. A statistically significant difference immediately at the end of day 10 of US exposure was found for all parameters measured, except NOR diameters of the stratum basale cells. All the measured parameters, except NOR numbers of fibroblasts in the dermis, returned to normal (control) values by day 74 indicating that the therapeutic effect of US at the given characteristics last at least longer than 46 d regarding thickness of the stratum corneum, longer than 32 d regarding NOR numbers in the cells of the stratum basale, and NOR diameters in the cells of the stratum basale, and longer than 74 d regarding NOR numbers in fibroblasts. The results presented herein suggest that there is a correlation between the longevity of ultrasound effect and the alteration of AgNORs parameters and their characteristics. Considering the longer duration of reversible alterations in our study, it would be reasonable to determine the effectiveness of ultrasound by AgNOR counting.

Key words: dog, skin, ultrasound, AgNOR proteins.

Material and Methods

The study was performed according to the Helsinki Convention for the use and care of animals.

A total of 42 adult dogs of both sexes, were used and divided into seven equal groups. A depilatory agent was used on either right or left side of the chest of each animal. Only one side of the chest was used for therapeutic US treatment. All the dogs except the controls were exposed to US treatment (0.5 W/cm², 1.0 MHz, pulsed 2 ms on and 8 ms off for 5 min daily) for 10 d.
Fig. 1. AgNOR (arrows) in the nuclei of fibroblasts in the dermis. Bar: 10µm, AgNOR staining.

Using a therapeutic US machine (Mettler Model 705 sonicator; Mettler Electronics Corp.), a 5 cm² piezoxide transducer (round contact area) was applied to the skin through ultrasound gel as a coupling agent. Full thickness skin samples were taken from the centre of the circular area in group 1 at the end of US application and in groups 2, 3, 4, 5, and 6 at 4, 18, 32, 46, and 74 d following the cessation of US application. In the group 7, full thickness skin samples were taken after the application of a depilatory agent to the both sides of the chest of six dogs that served as a true control.

Paraffin embedded 4 µm thick sections were dewaxed in xylene and rehydrated through ethanol series to deionised water. The silver staining solution was prepared according to the method described by Howat et al., (12). The sections were incubated in the dark with the staining solution for 40-45 min, washed in running deionised water, slightly counterstained with 1% methyl green, and then dehydrated in ethanol series, cleared in xylene, mounted and visualised as black dots (AgNOR) under the optical microscope, using a X100 oil immersion lens (Fig. 1). A total of 100 cells were counted in each microscopic specimen. Two diagonal measurements were also performed with a linear ocular micrometer on the NOR diameters of the cells of the stratum basale. ANOVA and Student's t test was used for statistical analysis and P<0.05 was considered statistically significant.

Results

Any unexpected clinical and histological changes were not observed in the skin through the experiment. There was a decrease in the stratum corneum thickness observed immediately after the end of 10 d US exposure. The decrease continued until the 46th d with statistically significant differences (P<0.05) and returned to the control level on the 74th d (Table 1).

Fibroblasts had oval or elongated nuclei with one or two distinct nucleoli. AgNORs were seen as oval-round shaped and brown-black coloured dots in the cell nuclei. Their numbers changed from 1 to 4, and they were mainly located eccentrically; at least one of them adjacent to the nuclear envelope (Fig. 1). Stratum basale cells contained indented nuclei with one or two prominent nucleoli. Their AgNORs displayed quite similar histological characteristics to those of fibroblasts. There were no definite changes in both location and shape of AgNORs among the US treated groups.

Starting at the end of US exposure, NOR numbers in the cells of the stratum basale decreased, and continued to decrease with statistically significant difference (P<0.05) until the 46th d compared to the control. Although it was below the control level, a rise was observed at the 74th d with no statistically significant difference compared to the control. Statistically significant difference (P<0.05) in the NOR numbers in the cells of the stratum basale was also observed at the end of 46th d and at the end of 4th d following cessation of US exposure, compared to the 10th d (Table 1).

No statistically significant differences were observed regarding the mean diameters of the NORs in the stratum basale cells at the end of 10 d US exposure and on the 4th and 18th d following the cessation of US application despite existence of a continuing decrease compared to the control. There was a statistically significant difference (P<0.05) for the decrease on the 32nd d compared to the control, 10th, 4th, and 74th d values following US cessation. A statistically insignificant rise was observed at the end of the 46th d compared to the control. Return to the control level with no significant difference was seen on the 74th d compared to the control (Table 1).
Table 1
Changes of cell parameters at the end of day 10 of US exposure and on days following the cessation of US exposure

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>End of 10 d US application</th>
<th>Days following the cessation of US exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>Thickness of ( \text{stratum corneum} ) (( \mu \text{m} ))</td>
<td>41.46±4.73(^a)</td>
<td>28.21±4.55(^b)</td>
<td>27.47±6.33(^b)</td>
</tr>
<tr>
<td>NOR number of cells of ( \text{stratum basale} )</td>
<td>3.50±0.45(^a)</td>
<td>2.01±0.20(^b)</td>
<td>2.03±0.25(^b)</td>
</tr>
<tr>
<td>NOR diameter of cells of ( \text{stratum basale} ) (( \mu \text{m} ))</td>
<td>2.65±0.41(^a)</td>
<td>2.50±0.29(^a)</td>
<td>2.45±0.30(^a)</td>
</tr>
<tr>
<td>NOR number of fibroblasts in the dermis</td>
<td>3.25±0.40(^a)</td>
<td>2.08±0.27(^b)</td>
<td>2.11±0.24(^b)</td>
</tr>
</tbody>
</table>

Different superscripts (a-d) on the same line denote statistically significant differences (\(P<0.05\)).

NOR enumeration of fibroblasts in the dermis showed a statistically significant decrease (\(P<0.05\)) at all time intervals compared to the control. The decreased values on the 18\(^{th}\), 32\(^{nd}\) and 46\(^{th}\) d were significantly different (\(P<0.05\)) compared to that of the 10\(^{th}\) d of US exposure and the 4\(^{th}\) d thereafter. Significant differences (\(P<0.05\)) were also observed in the values at the end of 74\(^{th}\) d compared to the 18\(^{th}\), 32\(^{nd}\), and 46\(^{th}\) d values. There were significant differences (\(P<0.05\)) on the 46\(^{th}\) d compared to the 10\(^{th}\) d of US exposure, and the 4\(^{th}\) and 18\(^{th}\) d thereafter.

Discussion

The presented study defines the possibility of using AgNOR enumeration as a biological counter to predict biological behaviour of skin subjected to therapeutic ultrasound application in the dog.

It was demonstrated that all the parameters measured, except the NOR diameters of \( \text{stratum basale} \) cells, showed statistically significant differences immediately at the end of 10 d US exposure. Our study was designed in such a way that the control group was a true control (non-treatment) group, not the one receiving placebo as inactive ultrasound because most studies revealed that there was no differences between groups treated with active ultrasound and placebo (sham) ultrasound (15, 23). Thus, the results of this study support those that found out significant differences between the control and experimental animals at the end of 10 d US exposure, based on AgNOR counting. All the measured parameters, except the NOR numbers of fibroblasts in the dermis, returned to normal \( \text{i.e.} \) to control values by day 74 indicating that the therapeutic effect of US at the given characteristics lasts at least longer than 46 d regarding thickness of the \( \text{stratum corneum} \), longer than 32 d regarding NOR numbers in the cells of the \( \text{stratum basale} \) and NOR diameters in the cells of the \( \text{stratum basale} \), and longer than 74 d regarding NOR numbers in fibroblasts.

As Saad \textit{et al.} (20) demonstrated short-lived changes of blood biochemical parameters and lesions in the liver \textit{in vivo} following 0.75-1.25 W/cm\(^2\) ultrasound application for 5 min. Regarding the AgNOR counts at the tissue level, our results indicate that although reversible, the alterations have longer duration.

The use of AgNOR counting has been increasing in other areas than the differentiation between malignant and benign lesions in cancerous patients. Altered changes in AgNOR count were reported in methylythiouacril induced thyroid proliferations (21), in normal oral buccal mucosa epithelium \textit{versus} leukoplakia and squamous cell carcinoma (14), and in normal \textit{versus} diseased stomach mucosa (17).

Physiologically no difference was observed for AgNOR count between the male and female 6-month-old Kangal breed Anatolian shepherd dogs (22). AgNOR decrease is related to slower cell doubling time (3). Decreased AgNOR was found in healthy mucosal epithelium subjected to continuous or intermittent compressive pressure through experimental denture bases (11). In the case of these studies showing a decreased number of AgNORs, the findings of our study also showed
decreased AgNORs in the skin of dogs exposed to US at the given characteristics.

The results presented herein suggest that there is a correlation between the longevity of ultrasound effect and the alteration of AgNOR parameters and conditions of the US applied in the study. Considering the longer duration of reversible alterations in our study, it would be reasonable to determine the effectiveness of ultrasound therapy through AgNOR counting. Underlying biological and physical mechanisms with variable determinants of ultrasound need to be fully investigated before a scientific basis is established for how long and how many days it should be applied for different tissues in order to have desirable conclusions, taking into account that AgNOR number varies among different cell types and tissues and effects of US application depends on the intensity, duration, type of application, and type of tissue.

References