THE EFFECT OF AD
BIOPHYTOMODULATORS ON BONE
HEALING IN ANIMALS

SUMMARY OF THE Ph.D. THESIS

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SUMMARY

Complementary medicine and alternative medicine are terms that define practices and products different from conventional, allopathic medicine.

Examples of alternative medicine include homeopathy, naturopathy, chiropractic and acupuncture. Complementary medicine is the alternative medicine used along with conventional medical treatments, "complementing" allopathic treatment. (Ernst, 1995)

There isn’t a complete definition, widely accepted alternative medicine (CAM Comitee, 2005). In the past 30 years research and practice in complementary and alternative medicine have evolved substantially. Acceptance by allopathic practitioners of these techniques and recognition of their have influenced research in the field (Gaboury et al., 2012).

Approximately 50% of the population in developed countries used a type of complementary or alternative medicine (Ernst, 2003, Barnes et al, 2004, Astin, 1998, 2000).

Alternative medicine includes a wide range of treatments and practices, the most common methods can be divided into five classes: biological based approach, energy therapies, alternative medical systems, musculo-articular manipulation, mind-body therapies.

Energy therapy is intended to influence energy fields (bio-fields), which supposedly enclose the entire body. National Center for Complementary and Alternative Medicine in the U.S. has distinguished two types of energy medicine, one that uses the "true", detectable energy fields and other that manipulates "subtle" energies, currently undetectable / unmeasurable (http://nccam.nih.gov/).

Insofar as the subtle energy associated properties are not overlapping the physical energy, it is difficult to scientifically prove the existence of such energy. Therapies that claim to use, manipulate or modify immeasurable energies are among the most controversial branch of alternative medicine.

The studies included in this paper were made between 2009 and 2013, and had as main objective the assessment of the stimulating effects of AD-DIEE biophytomodulators on bone healing, using experimental studies.
This paper is structured in two parts, the first part entitled "State of the art" covers 25 pages, and the second, entitled "Personal contributions" has 90 pages. This study contains 119 figures and 25 tables.

Chapter I, “State of the art” summarizes the current state of knowledge and extends over 6 chapters, which describe as follows: a general overview on bone tissue, mechanisms involved in fracture healing, experimental fractures and bone defects described in literature, how the process of fracture healing can be assessed, techniques used in complementary and alternative medicine and energetic medicine and other studies that included the use of AD-DIEE Biophytomodulators.

The second part of the thesis contains the personal contributions and contains four chapters. This part of the thesis describes the objectives, materials and methods used to obtain results that will confirm or refute the therapeutic properties of AD-DIEE Biophytomodulators. Presentation of the experimental part is followed by an assertion of general conclusions, recommendations, and literature cited. In the present study 102 references were cited.

Chapter II, “Histopathological studies on the reparative process in bone defects in sheep using AD-DIEE biophytomodulators” shows a histopathological study in sheep. The bone healing process in sheep treated with AD-DIEE devices were compared to those in a control group.

Materials and methods

The research was conducted on 12 sheep (Ovis aries). Bone defects of the same size were surgically inflicted on the right tibial crest of the animals in the study.

Surgeries were performed under general anesthesia. After surgically uncovering the tibial crest, removal of bone fragments was performed using a dental drill, under cooling with saline.

The animals were divided into 2 groups: control (M) and treated with AD-DIEE biophytomodulators (BF). An AD-DIEE device was applied in each animal in group BF, using sutures, on the medial side of the tibia, near the incision.
Evaluation of results was done by harvesting the callus in the defects, with histological examination made at 7, 14 and 21 days after the surgeries.

Harvested parts were histologically prepared. Five μm thick sections, stained with Masson Trichromic method, modified by Goldner, were examined under a microscope Olympus BX41, equipped with digital camera.

**Results and discussions**

At first harvest, 7 days after the surgeries, in M1 we noted ongoing repair processes, but in relatively early stage, a large number of active fibroblasts and fine collagen fibers. In the sample from the BF group, repair processes were present in a more advanced stage, compared to the control group, with significantly higher number of newly formed capillaries.

At 14 days after surgery, in the control group there were a greater number of fibroblasts and capillaries, but in BF sample connective tissue was consolidated and bone trabeculae were present in different stages of organization and consolidation.

After 21 days, the control had repair processes represented only by relatively well-vascularized connective tissue. On the other hand, in the BF sample, bone proliferation was well underway, there was noted the presence of a significant number of osteoblasts and thicker bone trabeculae with tendency of expansion.

**Partial conclusions**

Reparative processes in the BF group were conducted with a higher speed compared to controls.

The sheep turned out not to be the most suitable study model in terms of histopathological investigations.

**Chapter III, “Biochemical studies on the reparative process in bone defects in rats using AD-DIEE biophytomodulators”,** describes an experimental model for the evaluation of the bone healing processes in rats, by creating defects in the compact bone of the femoral shaft and also has a biochemical study of bone healing in rats treated with AD-DIEE devices, as compared to a control group.

**Materials and methods**

The biological material used is made of 18 rats of the Wistar line. The bone defects we made, by adapting the technique described by Monofouet et al. (2010) in
mice. Under general anesthesia, the femur was surgically revealed. Bone defects were made with a dental drill with the diameter of 1.7 mm, under continuous cooling using saline. The skin was sutured in separate points.

The animals were divided into two groups, control (M) and treated with AD-DIEE biophyтомodulators (BF), and housed in cages model M3, made out of polycarbonate. For the BF group, 3 devices were applied on the cages, according to the manufacturer.

Evaluated biochemical parameters were serum alkaline phosphatase, serum proteins, ionized calcium, total calcium and serum osteocalcin. There were collected 2 ml of blood from each animal from the orbital sinus, as follows: two days prior to surgery (T0), 14 days after surgery (T1) and at 21 days after surgery (T2), and the results were interpreted statistically.

### Results and discussion

Biochemical test results are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>BF</td>
<td>M</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>292.88±98.19</td>
<td>243.66±55.27</td>
<td>305.66±84.49</td>
</tr>
<tr>
<td>OC (ng/ml)</td>
<td>1.24±1.23</td>
<td>1.41±1.05</td>
<td>1.75±1.62</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.9±0.4</td>
<td>2.81±0.16</td>
<td>2.77±0.14</td>
</tr>
<tr>
<td>Ionized Calcium (mmol/l)</td>
<td>1.19±0.056</td>
<td>1.21±0.029</td>
<td>1.26±0.042</td>
</tr>
<tr>
<td>Total proteins (g/dl)</td>
<td>9.21±0.647</td>
<td>9.12±0.638</td>
<td>8.32±0.417</td>
</tr>
</tbody>
</table>

There was a statistically significant increase in ALP values in group BF compared with group M, from T0 to T1 and then a significant decrease between T1 and T2.

Komenou et al. (2005) showed that the absence of a significant variation of alkaline phosphatase was correlated with a failure in fracture healing. An increase in
alkaline phosphatase first 10 days after the operation has been linked to the formation of bony callus, but maintaining the high values of ALP at two months postoperative was related to the formation of a hypertrophic callus.

Sousa et al. (2011) also correlated elevated ALP in dogs with adequate healing of bone fractures.

Thus, we can say that the elevated alkaline phosphatase, recorded at BF group at 14 days after surgery, suggested an increased osteoblastic activity compared with controls.

Serum osteocalcin comes both from osteoclast activity and osteoblasts, so it is now seen as a marker of bone turnover, not a specific marker of bone formation (Clarke, 2008). Changes in osteocalcin values obtained during the study were not statistically supported.

In our study we found that total serum calcium levels decreased, having an evolution inversely proportional with the levels of alkaline phosphatase.

Two weeks postoperatively, there was a slight decrease in serum calcium, but with an increase in the ionized calcium fraction, necessary for the impregnation of newly formed bone matrix.

We found significant changes in ionic calcium values in both groups, from T0 to T1. At T2, BF ionized calcium values were still higher than at T0, the difference being statistically supported. This difference is consistent with the need for ionized calcium, for the mineralization of the newly-formed callus.

Decreases in total protein levels in animals in group BF, from T0 to T1 and from T1 to T2 was statistically supported. The decrease of the mean value of total protein level in animals from the control group was statistically supported between T0 and T1.

In the BF group, the decreased serum protein level registered at T2 (21 days), may be associated with a greater amount of newly formed callus in this group (chapter VI).

Partial conclusions

Variations in protein, alkaline phosphatase and ionized calcium levels suggest a slightly more intense osteoblastic activity in the animals in group BF.
Chapter IV "Imaging studies on the reparative process in bone defects in rats using AD-DIEE biophytomodulators" summarizes the results obtained with digital radiography and computed tomography.

Materials and methods

There were used 12 Wistar rats. To achieve bone defects the same protocol described in Chapter III was used.

The animals were divided into two equal groups, control group (group M) and the group treated with AD-DIEE biophytomodulators (group BF).

Medical equipment used for imaging studies (Table 2) was represented by a Villa digital x-ray machine and NewTom 3G Scanner.

The images obtained were analyzed with NNTViewer (for CTs) and ImageJ (for x-rays).

Table 2

<table>
<thead>
<tr>
<th>Imaging investigations carried out in the study</th>
<th>1 week postoperatively</th>
<th>2 weeks postoperatively</th>
<th>3 weeks postoperatively</th>
<th>6 weeks postoperatively</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RX</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</table>

Results and discussion

CT

At 3 weeks postoperatively, the average size of the defect decreased to 1.22 mm in control and 1.06 mm in the treated animals.

At six weeks post-surgery, the bone defect is hardly visible because it almost completely filled with newly-formed callus, significantly reducing the size of the defects in both groups of animals by an average of 0.82 mm in control and 0.72 mm in the BF group. Discontinuity observed in the images was a difference in bone density, as demonstrated by histopathological investigations of the next chapter. The defect is filled with newly-formed callus and is in the process of calcification and remodeling.
RX

When comparing the color intensity between the defect and an identical area of compact bone adjacent to the defect, the results obtained were statistically supported, something which shows that selection for analysis at the level of defects were made properly, both in images at 3 weeks and 6 weeks post-operatively. Average color intensity was higher in image of defects from group BF, but this difference was not statistically supported.

At 6 weeks postoperatively, when calculating the difference between the intensity of color in the defect and the adjacent compact bone, these differences were smaller for BF group compared with controls. This demonstrates that in the BF group the intensity of the color of the defect is closer to the one of compact bone, which is statistically supported (p = 0.046).

Partial conclusions

Imaging investigations gave us clear view of the dynamic of the reparative processes carried out in the bone defect.

At 3 and 6 weeks postoperatively, CT images showed that the bone defects were reduced in diameter. Average defect diameter was smaller in group BF, but the difference compared to the control group was not statistically supported.

Radiological image analysis in ImageJ showed a higher intensity in the color of bone defects in BF group compared to the M group.

Chapter V, named ”Histopathological studies on the reparative process in bone defects in rats using AD-DIEE biophytomodulators” is the last chapter of the research and presents the results of the histopathological study in rats.

Materials and methods

The study included 18 Wistar rats. To achieve the bone defects, the same protocol described in Chapter III was used.

The animals were divided into two equal groups, control group (group M) and the group treated with AD-DIEE biophytomodulators (group BF).

For histological investigation, the femoral shafts including the bone defect were harvested at 2, 4 and 6 weeks after surgery. The animals were euthanized according to
national and european regulations. Samples were processed histologically and stained with Masson Trichrome technique or hematoxylin-eosin.

Results and discussion

The histopathological images show that in all stages of the study the bone healing processes are more advanced in the BF group compared with controls.

At 6 weeks postoperatively, in the BF group, the continuity of the bone wall was provided by a continuous layer of bone, which disrupts communication between the exterior and the medullary canal. On the inner side of the bone, cancellous bone tissue, composed of fine bone trabeculae, proliferated, strengthening the bone wall. This aspect was not found in the control group.

Partial conclusions

Reparative processes were more advanced in group BF at all stages of the study.

The AD-DIEE biophytomodulators have been shown to have a beneficial effect in stimulating the proliferation of bone to some extent, without any great differences when compared to the control.

Chapter VI lists the general conclusions that can be drawn from the partial conclusions of each chapter of the research.

References


7. **Gaboury Isabelle, Karine Toupin April, Marja Verhoef** (2012) A qualitative study on the term CAM: is there a need to reinvent the wheel?, *BMC Complementary and Alternative Medicine*, 12:131


