COMPARATIVE STUDY ON THE EFFECT OF NANO-HYDROXYAPATITE AND THEOBROMINE ON HEALING OF EXTRACTION SOCKET IN ALBINO RATS (HISTOLOGICAL AND IMMUNO-HISTOCHEMICAL STUDY)

Sherif Mohsen Al Bahrawey*, Ahmed Mahmoud Halawa**, and Hend El-Messiry***

ABSTRACT

Background: Dental extraction is one of the most widely performed dental procedures. Socket healing involves tissue repair and regeneration. Different types of materials are used to aid and fasten the healing process such as nano-hydroxy appetite.

Objectives: The aim of the present study was to compare the healing potential and potencies for new bone formation of nano-hydroxyapatite crystals and theobromine powder in the healing of extraction Socket in Male Albino rats.

Methods: Thirty-eight male Albino rats were used this experiment. Lower left first molar was extracted from each rat. The rats were equally divided into three groups. Each group was subdivided into two subgroups (After one week and after two weeks) from receiving the treatment. control group which didn’t receive any treatment, n-HA group which received –HA and theobromine group which received theobromine. Samples were stained with H&E, Masson trichrome and Beta-catenin then assessed under light microscope. One-way ANOVA followed by Tukey’s post hoc test was used to analyze data.

Results: H&E section showed that theobromine group has more potential in inducing new bone formation than n-HA group. Masson trichrome stain: revealed that theobromine group induced mature bone formation compared to n-HA group. Beta-Catenin stain: All groups showed negative reactions except theobromine group 3B and n-HA group 2B.

Conclusions: Nanohydroxy apatite crystals and theobromine enhance the socket healing, theobromine has superior effect compared with the nanohydroxy apatite. the effect of the treatment material is more obvious in the second week of socket healing.

KEYWORDS: Socket healing; nano-hydroxy appetite; theobromine.
INTRODUCTION

Tooth extraction starts a sequence of healing procedures that include soft (such as gingiva and the periodontal ligament) and hard (such as alveolar bone) tissue. Characterizing the tissues involved in the healing process of extraction sockets has been made easier by studying the biological processes that occur throughout the healing process and their temporal order in various animal models.\(^1\) (Farina and Trombelli, 2011).

Healing of the hard and soft tissues after extraction goes without a delay. On the other hand, the alveolar defect caused by tooth extraction will only be partly repaired. Regardless of the kind of tooth, resorption of the alveolar ridges occurs in tandem with bone development into the socket, resulting in notable alterations in its proportions.\(^2\) (Couso-Queiruga et al., 2021).

Different materials are used to optimize the bone healing process such as autogenous (patient own bone), allogeneic (dead bone collected from a corpse, then processed using a freeze-dry method to extract the water via a vacuum), xenogeneic (bone taken from animals), and alloplastic (synthetic bone grafts made from hydroxyapatite or made from bioactive glass) bone grafts. Other materials like platelet-rich plasma, platelet-rich fibrin, bone morphogenetic protein, (n-HA) crystals, Emdogain, and cell therapy can also be used\(^3\) (Kantharia et al., 2014).

(Kantharia et al., 2014)\(^3\) stated that n-HA presents an excellent bioactivity, angiogenic properties, no toxicity, and absence of inflammatory or antigenic reactions. It also can be used as filler for reinforcing restorative glass ionomer cement and restorative composite resin, desensitizing agent post bleaching, for treating early carious lesions and as a remineralizing agent in toothpastes. Application of n-HA in gel form after extraction of mandibular incisors of albino rats was discovered to stimulate the alveolar ridge bone creation more than 50% compared with the control group.\(^4\) (Pan et al., 2020).

Theobromine may potentially promote skeletal growth since it may stimulate the creation of HA during tooth development. It was hypothesized that this natural osteoanabolic supplement might be a great option for pregnant women, lactating mothers, and the early stages of postnatal growth. In order to assess the impact of THB on human osteoprogenitors, researchers conducted experiments using primary human bone marrow mesenchymal stem cells (hMSCs). They exposed the hMSCs to THB in a laboratory setting and then observed the effects by performing osteogenic assays. The results indicated that exposure to THB led to an enhanced rate of osteogenesis and mineralization by the hMSCs\(^5\) (Clough et al., 2017). Yet not many studies were done to grade THB effect in bone healing in comparison with n-HA crystals. Based on this research, we aimed to compare between n-HA and THB in the efficacy on extraction sockets healing in Albino rats.

MATERIALS AND METHODS

I- Materials used in the present study are:
A- Animals: Thirty-eight male Albino rats weighting (200-250 gram) were used in this study.
B- Nano-hydroxyapatite crystals(n-HA): purchased from Sigma-Aldrich Company.
C- Theobromine (THB): Purchased online from Barlower’s Herbal Exlixirs company Florida in the form of capsule (each of 400 mg).

II- Methodology:

I- Extraction process: Rats were sedated by ketamine 10 mg/ml concentration and the mandibular left first molars of each rat in all groups were extracted using needle holder. Post-
operative systemic antibiotic (100 mg/kg body weight given intramuscularly once daily for seven days) and anti-inflammatory (diclofenac sodium 15 mg/kg body weight administered intramuscularly twice daily for three days).

2- Grouping: The lower left first molar was extracted in all the 38 rats used in this study. The rats were divided into the following groups; Group I (control group), Group II (n-HA group), Group III (THB group). Each contained 14 rats except control group contained 10 rats.

**Group I (Control group):** Rats have not received any topical treatment into the extraction socket.

**Group II (n-HA group):** Nano-hydroxy apatite crystals gel was applied to fill the extraction sockets directly after the extraction procedure.

**Group III (THB group):** Theobromine powder gel was applied to fill the extraction sockets directly after the extraction procedure.

Each group is subdivided into 2 subgroups according to the time of rat scarification as follow:

**Subgroup A:** Rats were sacrificed after 1 week from the extraction date.

**Subgroup B:** Rats were sacrificed after 2 weeks from the extraction date.

3- Sample collection:

At the end of each specified period, the rats of the corresponding subgroups were sacrificed separately by cervical dislocation and their heads were immediately dissected to obtain the mandibular molar area and caresses were disposed of by incinerator. Specimens were processed for routine histological, histochemical and immuno-histochemical (Beta-Catenin) examination under light microscope.

**Statistical analysis**

Data were populated and statistically analyzed. The data were described as mean and standard deviation. One way ANOVA test was used to compare between groups. Post-hoc tukey was used to compare between each two groups.

**RESULTS**

The present study compared the effect of two different materials on extraction socket healing; n-HA and THB by routine histological examination and immuno-histological examination.

1- Results of the Hematoxylin and Eosin stain:

**Group 1 (control group):**

**Subgroup 1A (1 week):**

Examination of extraction sockets sections of subgroup1A showed that the extraction socket was filled with granulation tissue which was consisted of lymphocytic infiltration, loose collagen fibrils and cellular elements (fibroblasts, plasma cells, macrophage) embedded in a primitive connective tissue. The extraction socket wall appeared to be lined by many regular active osteoblasts intermingled with osteoclasts in Howship’s lacunae. No evidence for new bone formation was found.

![Fig. (1) Photomicrograph of subgroup 1A showing regular active elongated osteoblast (black arrow) and occasional osteoclasts (red arrows) (H&E original magnification x400)](image-url)
**Subgroup 1B (2 weeks):**

Examination of the extraction socket after two weeks showed that the socket was filled with dense fibrous connective tissue, dense inflammatory cell infiltration especially at the periphery of the socket, and woven bone trabeculae. These woven bone trabeculae that were detected in the central region as well as at the socket margins enclosing numerous round plump osteocytes that inhabited in wide lacuna. This newly formed bone trabeculae enclosed apparently wide marrow cavities with osteoblast riming them. At the lateral margin of the socket. Newly formed Haversian canal with osteoblast riming and a reversal line separating the newly formed bone from old bone were seen.

**Group 2 (n-HA group):**

**Subgroup 2A (1 week):**

Examination of the extraction sockets of this subgroup showed irregular socket walls. Fibrous connective tissue surrounding the socket bone margins with a remarkable decrease in the number of inflammatory cells compared with the control group. Evident new bone development was seen. The newly formed bone enclosed marrow cavities.

**Subgroup 2B (2 weeks):**

Specimens of the extraction socket of this subgroup revealed obvious new woven bone trabeculae radiating from the socket fundus to the socket center n-HA remnants.

**Group 3 (Theobromine group):**

**Subgroup 3A (1 week):**

Light microscope examination of this group revealed new woven bone formation with irregular bone trabeculae. The neighboring connective tissue showed High vasculature. Formation. Trabeculae of woven bone also showed numerous plump osteocytes in wide lacunae in addition to the presence of developing blood

---

**Fig. (2) Photomicrograph of subgroup 1B showing the extraction socket filled with organized dense fibrous connective tissue. Dense inflammatory cell infiltrations with fibrous connective tissue surrounding (red arrows) and new bone spicule formation in the socket core (black circle). New woven bone was formed in the fundus of the socket (black rectangle). (H&E original magnification x200).**

**Fig. (3) A photomicrograph of subgroup 2A showing the socket filled with dense fibrous connective tissue (red arrow), granulation tissue (blue arrow) and new woven bone formation (black circle) (H&E original magnification x200).**

**Fig. (4) Photomicrograph of subgroup 2B showing the extraction socket with new woven bone trabeculae extended to the socket (red arrows) and n-HA remnants (blue arrow) (H&E original magnification x100).**
vessels. Vascular granulation tissue was detected at the remained central area of the socket.

**Subgroup 3B (2 weeks):**

Specimens of the extraction socket of this subgroup revealed the newly formed bone that were filling the socket took the lamellar pattern. This new bone contains numerous spindle shaped osteocytes occupying wide lacunae which surround circumferentially the developing osteons and marrow space that are lined by active elongated osteoblast. An area of incomplete healing was seen containing dense fibrous connective tissue with little inflammatory cells presence. A reversal line was seen dividing the newly created bone from the old previous one.

**Group 1 (control group)**

**Subgroup 1A (1 week):**

Examination of extraction sockets sections of subgroup1A showed that the extraction socket was filled with granulation tissue which was formed of newly formed loose collagen fibrils appearing as blue fibers and cellular elements.
Subgroup 1B (2 weeks)

Examination of the extraction socket of this group that was stained Masson trichrome showed that the socket was filled by dense fibrous connective tissue containing apparently seen mature fibers in relation to subgroup 1A, dense inflammatory cell infiltration was seen at the periphery.

Group 2 (n-HA group):

Subgroup 2A (1 week):

Examination of the extraction sockets of this subgroup stained with Masson trichrome showed fibrous connective tissue with a less predominance of inflammatory cells in comparison with the control group. There are trabeculae of new woven bone observed enclosing wide osteocyte lacunae.

Subgroup 2B (2 week):

Masson trichrome examination of the sockets after extraction of this subgroup revealed obvious new woven bone trabeculae in the socket center surrounded by fibrous connective tissue which consist of newly formed collagen fibers and cellular aggregations of fibroblasts and inflammatory cells. The newly formed bone contained new round plump osteocytes.
**Group 3 (Theobromine group):**

**Subgroup 3A (1 week):**

Light microscope examination of this group stained with Masson trichrome revealed presence of mature collagen fibers. Apparent inflammatory cells, fibroblasts, mature collagen fibers and hylanized matrix were seen.

![Fig. (13) A photomicrograph of the socket in subgroup 3A showing inflammatory cells (blue arrow), fibroblasts (red arrow), mature collagen fibers (yellow arrow) and hyalinized matrix (black arrow) (Masson Trichrome stain x400).](image)

**Subgroup 3B (2 weeks):**

Masson trichrome examination of the sockets after exaction of this subgroup revealed newly formed lamellated compact bone in the extraction space connected old mature compact bone. This new bone contains numerous spindle shaped osteocytes present in wide lacunae.

![Fig. (14) A photomicrograph of the socket in subgroup 3B showing newly formed mature bone with areas of compaction (arrow) (Masson Trichrome stain x200).](image)

**III- Results of the Beta-Catenin stain:**

**Group 1 (control group):**

Examination of extraction sockets sections of subgroup 1A (control group of 1 week) showed negative reaction in the cells. Likewise, the extraction sockets sections of subgroup 1B (control group of 2 weeks) showed no observed positive reactions in the cells covering the newly formed bone.

![Fig. (15) A photomicrograph of the socket in subgroup 1A showing negative reaction in the cells (Beta catenin x400).](image)

**Group 2 (n-HA group):**

Examination of extraction sockets sections stained with Beta-Catenin of subgroup 2A (n-HA group of 1 week) showed negative reaction in the cells. While the extraction sockets sections of subgroup 2B (n-HA group of 2 weeks) showed positive immunohistochemical reaction in some cells lining the bone at the bone.

![Fig. (16) A photomicrograph of the socket in subgroup 2A showing negative reaction (Beta catenin x400).](image)
Fig. (17) A photomicrograph of the socket in subgroup 2B showing positive immunohistochemical reaction in some cells lining the bone at the bone (Arrows) (Beta catenin x400).

**Group 3 (Theobromine group):**

Examination of extraction sockets sections stained with Beta-Catenin of subgroup 3A (Theobromine group of 1 week) showed negative reaction in the cells. The extraction sockets sections of subgroup 3B (Theobromine group of 2 weeks) showed occasional positive reaction in the newly entrapped osteocytes.

Fig. (18) A photomicrograph of the socket in subgroup 3B showing occasional positive reaction in the newly entrapped osteocytes (arrow) (Beta catenin x400).

**Statistical results**

Statistical results showed that Within Week 1: n-HA & THB Groups (8.18±1.24; 20.69±4.14 respectively) showed significance increase compared to that in Control Group (2.65±0.47) (p=<0.001, <0.001 respectively). Also THB Group (20.69±4.14) showed significance increase compared to that in n-HA Group (8.18±1.24) (p=<0.001). Within Week 2: n-HA Group (10.96±2.19) showed significance increase compared to that in Control Group (3.17±0.93) (p=<0.001) while THB Group (1.75±0.45) showed non significance compared to that in Control Group (3.17±0.93) (p=0.07). THB Group (1.75±0.45) showed significance decrease compared to that in n-HA Group (10.96±2.19) (p=<0.001). Within Control group: After Week 2 subgroup (3.17±0.93) showed non significance compared to that in After Week 1 subgroup (2.65±0.47) (p=0.12). Within n-HA group: After Week 2 subgroup (10.96±2.19) showed significance increase compared to that in After Week 1 subgroup (8.18±1.24) (p=0.003). Within THB group: After Week 2 subgroup (1.75±0.45) showed significance decrease compared to that in After Week 1 subgroup (20.69±4.14) (p=<0.001). Table (1)

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>n-HA group</th>
<th>THB group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After Week 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc</td>
<td>2.65±0.47</td>
<td>8.18±1.24</td>
<td>20.69±4.14</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P1=&lt;0.001*</td>
<td>P2=&lt;0.001*</td>
<td>P3=0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After Week 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc</td>
<td>3.17±0.93</td>
<td>10.96±2.19</td>
<td>1.75±0.45</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P1=&lt;0.001*</td>
<td>P2=0.07</td>
<td>P3=&lt;0.001*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean±SD  SD: standard deviation  P: Probability  *: significance <0.05

Test used: One-way ANOVA followed by post-hoc tukey

P1: significance between Control & n-HA groups,  
P2: significance between Control & THB groups,  
P3: significance between n-HA & THB groups
DISCUSSION

Tooth extraction and the subsequent bone resorption impair the post extraction dental treatment. Therefore, the socket preservation aims to reduce bone loss is necessary. Many biomaterials for socket grafting have been reported in the literature (Jamjoom and Cohen, 2015; Anwandter et al., 2016) (6-7). Thus, this study was done to evaluate the possibility of enhancement of the healing of extraction socket. This was done by using two topical materials as post extraction, surgical complications may have serious consequences for patients as well as doctors. (Yamada et al., 2022) (8).

The selected materials that were used to encourage socket healing are Nanohydroxy apatite (n-HA) and theobromine (THB).

Regarding n-HA, it was selected as a possible socket healing promoting factor as it was reported by many authors that this material has high regenerative capacity (Chaves et al., 2012; Meimandi et al., 2013; Kattimani et al., 2016) (9) (10) (11), in addition to excellent bioactivity and angiogenic properties. It was also used as a remineralizing agent in many toothpastes (Kantharia et al., 2014) (3) and also was postulated that n-HA induced new bone formation and increased proliferation and metabolism of osteoblasts promoting superior osteoconductivity (De Tullio et al., 2019; Hakam et al., 2020) (12) (13).

Replacing the manufactured medications with natural therapeutic products was the main reason for the use of THB in this study. Bone healing such as in extraction socket - using plants and their extract was widely practiced because of the worries over the bad effects of conventional medicine (Tayung and Saikia, 2003; Moghadam et al., 2020) (14) (15). As far as we know, there is no previous research considering the direct effect of THB on the extraction socket healing in Albino rats for various periods of healing.

For evaluating the socket healing process in this study, Hematoxylin and Eosin (H&E) staining was used as it allows a general overview of the histological section. For further confirmation of bone maturation, masson trichrome staining was used due to its ability to differentiate between the new and mature bone (Hakam et al., 2020) (16).

The immunohistochemical marker used in the present work was β-catenin as it is necessary for osteoblast differentiation during bone formation in bone repair (Baht et al., 2015) (17). In the early period of bone healing B-catenin signaling facilitates the differentiation of osteoblasts. Moreover, many chemical elements can increase B-catenin signaling, such as lithium, which improves bone healing and increases bone density (Silkstone et al., 2008) (18).

In the current study extraction socket healing was examined after one and two weeks of the extraction because these periods are the most critical periods in the healing process. No bone healing starts during first week of the extraction only soft tissue healing occurs (Garlet et al., 2012) (19). Hence, we intended to evaluate the effect of n-HA and THB in the bone healing process at one & two weeks.

In the current study, the control group in 1-week (1A) revealed granulation tissue formation with no evidence of new bone deposition. The reason of the formation of this granulation tissue was explained by (Einhorn & Gerstenfeld, 2015) (20), who postulated that granulation tissue was formed due to secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha, bone morphogenetic proteins and interleukins (IL-1, IL-6, IL-11, IL-23). These cytokines attract macrophages, monocytes, and lymphocytes. The function of these cells is to remove damaged, necrotic tissue and secrete cytokines like vascular endothelial growth factor to promote the healing at extraction site. Moreover, Pan et al., (2020) (4) reported that after one week of extraction, only soft tissue healing occurred.

In contrast, the control group in 2 weeks (1B) the beginning of new bone formation was detected in the form of woven bone containing numerous osteocytes in wide lacuna. This finding was in accordance with
Machida et al., (2010) (21) who reported evidence of bone formation during the second week of healing when working on rat extraction sockets of right mandibular incisors.

In the present work, examination of the n-HA group after one week (2A) revealed fibrous connective tissue adjacent to the socket bone margins with a remarkable decrease in the number of inflammatory cells. These findings agreed with (Li et al., 2020) (22) who found that n-HA decreases the inflammatory markers such as tumor necrosis factor α thus decreasing the inflammation when placed within the osteoporotic rat femoral condyles that had bone defects.

In addition to the inflammatory cell infiltrate, there was an evidence of new bone formation in the extraction socket which was not observed in the corresponding subgroup in the control rats, indicating the osteogenic promoting effect of the n-HA. This coincides with Meirelles et al., (2008) (23) who evaluated the bone formation on titanium implants coated with n-HA particles in the rat tibia. Histological evaluation demonstrated significantly increased bone formation to the coated implants. The authors concluded that early bone formation is dependent on the nanosized HA features.

The new bone formation might be enhanced by n-HA as it could be used as an effective carrier of osteoinductive growth factors, which ensures osteoinductivity of n-HA via sustained release of the growth factor as explained by Miao et al., (2019) (24). Zhao et al., (2020) (25) also postulated that n-HA prolongs continued release of basic fibroblast growth factor which increases bone healing. This role of n-HA in providing the growth factors might explain the osteogenic influence because the growth factors are important for acceleration of bone regeneration (Zhang et al., 2017) (26).

In our work, examination of the n-HA group after two weeks (2B) revealed apparent new woven bone trabeculae radiating from the socket fundus to the socket center. The newly formed bone was bordered by numerous active osteoblasts as well as new round plump osteocytes. These findings coincide with (Zhao et al., 2016) (27) in his study who reported that osteoblasts adhesion and differentiation can be boosted by the help of n-HA.

Moreover, the n-HA group of the current week presented that new bone was connected to the old bone containing blood capillaries in addition to the presence of n-HA remnants. These findings were matched with Li et al., (2020) (22) who reported that the nano structure of n-HA increased the expressions of osteogenic genes and angiogenic genes thus increased angiogenesis potential. According to earlier research, this provides more evidence for the osteoconductivity and biocompatibility of n-HA (Tetsu et al., 2010) (28).

THB groups were examined in current study after one week (3A) and revealed new woven bone formation with irregular bone trabeculae. This was found in accordance to (Sarmadi et al., 2020) (29) who found that THB treatment in rats with induced osteoporosis largely increased serum insulin growth factor I and inhibited bone turnover to repair bone microarchitecture and strength.

The role of THB in bone growth could be explained by the enhancing effect of THB for production of growth factors as reported by Sumiyoshi et al., (2019) (30) who stated that dark chocolate consumption increased the nerve growth factor and THB levels in plasma. This supports THB potential and ability to promote bone healing based on the study of Grills et al., (1997) (31) that postulated that nerve growth factor increased bone healing in fracture sites which supports the current study outcomes.

Moreover, these findings were confirmed by Clough et al., (2017) (5) who postulated that The rate of osteogenesis and mineralization by hMSCs was enhanced by THB exposure. Additionally, a list of up-regulated mRNA transcripts that best matched
an expression of osteogenic tissue was produced as a consequence of THB exposure.

In another agreement with the present study the role of THB in osteogenic activity was reported by Spindel, (1984) (32) that THB (methylxanthines) increased serum corticosterone and beta-endorphin. Beta-endorphin plays a vital role in stimulating the thyroid gland for secretion of calcitonin hormone which plays two important roles during bone healing. First, analgesic effect of calcitonin that might be mediated by the increase of beta-endorphin stated by (Laurian et al., 1986) (33). Secondly, Calcitonin stimulates the production of new bone by upregulating Wnt10b expression in osteoclasts. (Hsiao et al., 2020) (28). Wnt10b is a protein which enhances bone formation by increasing osteoblast differentiation and proliferation (Bennett et al., 2007) (34).

The observed new areas of bone formation in the current study in the THB groups coincide with Yasuhiro et al., (2020) (35) who suggested that continuous THB consumption may boost bone density by encouraging osteoblast proliferation and differentiation and inhibiting osteoclast differentiation. In the current study the bone maturation was found to be more obvious in THB group this could be explained by the ability of THB to increase estrogen and androgen hormones (Kiyama.,2019) (36) and these hormones play the most important role in bone maturation (Satoh and Hasegawa., 2022) (37).

CONCLUSIONS

1. Nanohydroxyapatite crystals and theobromine enhance the socket healing.
2. Theobromine has superior effect compared with the nanohydroxyapatite
3. The effect of the treatment material is more obvious in the second week of socket healing.

REFERENCES


