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HISTOLOGICAL EFFECT OF HPC HORMONE ON BONE HEALING AFTER ITS APPLICATION ON SURGICALLY CREATED DEFECT

Running title: HPC Effect on Bone Healing in an Animal Model

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ABSTRACT

Bone is highly vascularized living connective tissue responsible for important metabolic and mechanical functions. Progesterone was first isolated in 1934 by groups investigating the endocrine function of the corpus luteum. Sixty adult male domestic rabbits were selected, with an average weight of 1750 g and an age range of 8 to 10 months. After the rabbit was anesthetized, an incision 4 cm in length was made in the left leg above the femoral bone, followed by dissection of the muscle, fascia, and periosteum to expose the femoral bone. Two holes were made with a round bur of 3 mm, and the space between holes is about 1 cm. Each hole is away from the edge of the femoral bone by 1 cm. In the control negative group, the two holes were lifted without filling with any material, while in the control positive group, the two holes were filled by placement of gel-foam pieces, and in the study group, the holes were filled with gel-foam, which was soaked in hydroxyprogesterone caproate. The study group showed a closed defect faster than the other two groups; in seven days, it showed a narrow defect bone site area between the edge of the old bone under its granulation tissue, connective tissue with high new bone formation, cartilaginous tissue, and well angiogenesis, with a significance of p-value 0.033. In conclusion, HPC accelerates bone healing in different time intervals by activating osteoblasts to form bone.

Keywords: HPC, Bone healing, Histopathological, Progesterone.

INTRODUCTION

Bone is a living, vital, dynamic, highly vascularized connective tissue, structurally complex, and heterogeneous (Morgan *et al.,* 2013; Almeida *et al.,* 2017) responsible for important metabolic (D'Mello *et al.,* 2017) and mechanical functions (Black and Tador, 2020; Blumer, 2021). It continues to remodel throughout the lifetime of an individual (Stevens, 2008).

Collagen-rich extracellular matrix (ECM) and elastic fibers attached to hydroxyapatite

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crystals comprise the structure of bone. Adult bone is constantly changing due to certain interactions between osteoblasts and osteoclasts (D'Aquino *et al.,* 2009). 1934 saw the first isolation of progesterone by a team studying the corpus luteum endocrine activity (Jewson *et al.,* 2020). Progesterone began to synthesize from diosgenin, extracted from the Japanese plant Dioscorea tokoro, and later from Dioscorea Mexicana (Taraborrelli, 2015). Rather than traditional recuperation, bone healing is a process of regeneration. Instead of scars, fresh, healthy bone tissue forms, as a result of this procedure. A variety of factors can influence the process of bone healing, which involves the production of new bone, angiogenesis, and soft tissue healing depending on the degree of disorder (Lisowska *et al.,* 2018).

Progesterone is an essential physiological element of the menstrual cycle, reproduction, and manufacturing of steroid hormones. The idea that progesterone is key to life is further supported by other physiological effects on the immune system and central nervous system, and the better of this hormone aids in a wide range of human health (Nagy *et al.,* 2021). The sterile, long-acting formulation of Hydroxyprogesterone Caproate Ester, a naturally occurring progestational hormone, in an oil solution for intramuscular application, is known as Hydroxyprogesterone Caproate (HPC) injection (Santa Ana, 2015). Its molecular weight is 428.60, and it has an empirical formula of C27H40O4. The chemical name for HPC is pregn-4-ene-3,20-dione, 17[(1-oxohexyl) oxy]. The crystalline powder known as hydroxyprogesterone caproate is white to creamy white in color (Shirley, 2018; Santa Ana, 2015).

It has been proposed that HPC might have a positive effect on bone healing. This study aimed to assess the histopathological effect of HPC on rabbit bone healing after its application.

MATERIALS AND METHODS

Animal Study-Ethical Statement

The study protocols were approved by the research ethics committee of the University of Mosul/College of Dentistry, REC reference number: UoM. Dent. 25/23. This study was held in the College of Dentistry, University of Mosul, Iraq, from May 2023 to September 2023.

Study Design

Sixty domestic adult male rabbits were selected; their average weight was 1750 gm, and their age ranged between 8 and 10 months. Each rabbit was given a 40 mg/kg ketamine injection (Paknejad *et al.,* 2007; Mahmood, 2022) intramuscularly in the thigh muscle, mixed with xylazine 4 mg/kg (Kilic, 2004; Mahmood, 2022). The weight of the rabbit was recorded using digital scales. After 5–10 minutes, the rabbit reflexes were checked to ensure that anesthesia was occured.

Surgical procedure

After the rabbit was anesthetized, animal hair was removed, and the area was cleaned with a povidone-iodine solution. The animal was put on its side at the surgical table. A 4 cm incision was made in the left leg above the femoral bone using a no. 15 blade, followed by dissection of the muscle, fascia, and periosteum to expose the femoral bone. Two holes were drilled using a low-speed hand piece with a round bur of 3 mm with irrigation of normal saline. The space between holes was about 1 cm and away from the edge of the femoral bone by the same distance. In the negative control group, the holes were left unfilled. In the positive control group, the holes were filled with a piece of gel-foam that is standardized for this purpose. In the study group, the holes were filled with gel-foam soaked in hydroxy-progesterone caproate (HPC).

Histopathological Examination

The preparation of the tissue specimen started with fixation, which is necessary to preserve the tissue by using an adequate volume of fixative solution formaldehyde. Demineralization in an aqueous solution followed by dehydration, and a clearing procedure (xylene solution) was done, and final staining was done using hematoxylin-eosin.

The histological scoring was done according to the criteria in Table (1). A computer package (Sigma Stat V12.0/SSYSTAT software) was used to conduct the histological criteria analysis. A total of 120 samples of 60 rabbits were examined blindly by two histopathologists.

Statistical analysis

Statistical analysis was presented as Means ± SE (standard error) and was analyzed by a one-way ANOVA test using Duncan's test among groups and periods. $P \leq 0.05$.

Table 1: Criteria for bone scoring histological sections (Alemi *et al.*, 2019).

Score	Histopathological Response Observed
0	None Newly formed vessels None to very minimal Numbers of fibroblasts None Osteoid (bone matrix) None Bone
1	Few Newly formed vessels A Numbers of fibroblasts Evidence Osteoid (bone matrix) Evidence of bone formation
2	Moderate Newly formed vessels Predominantly Numbers of fibroblasts Moderate Osteoid (bone matrix) Moderate bone cells
3	Extensive Newly formed vessels Fewer Number of fibroblasts Dense highly organized Osteoid (bone matrix) Extensive bone cells

RESULTS

All the histological results involve the scores of three groups, control negative, control positive, and study group, at four different times: 3, 7, 21, and 28 days. Table 2 represents the scores of all groups at all periods, mean values, and p-values.

Three days period:

Histological section of the rabbit's femoral bone over 3 days for the control negative group showed a wide defect area between the edges of the old bone with a large amount of inflammatory exudate, including inflammatory cells, while the control positive group showed a wide defect area between the old bone with a large number of inflammatory cells and high fibrinous exudate, while the study group showed a narrow defect bone area between the edges of the old bone with a large number of granulation tissue with less inflammatory cells infiltrate and rests of study material present as seen while the study group showed a narrow defect bone area between the edges of the old bone with a large number of granulation tissue with less inflammatory cells infiltrate and rests of study material present as seen in Figure (1).

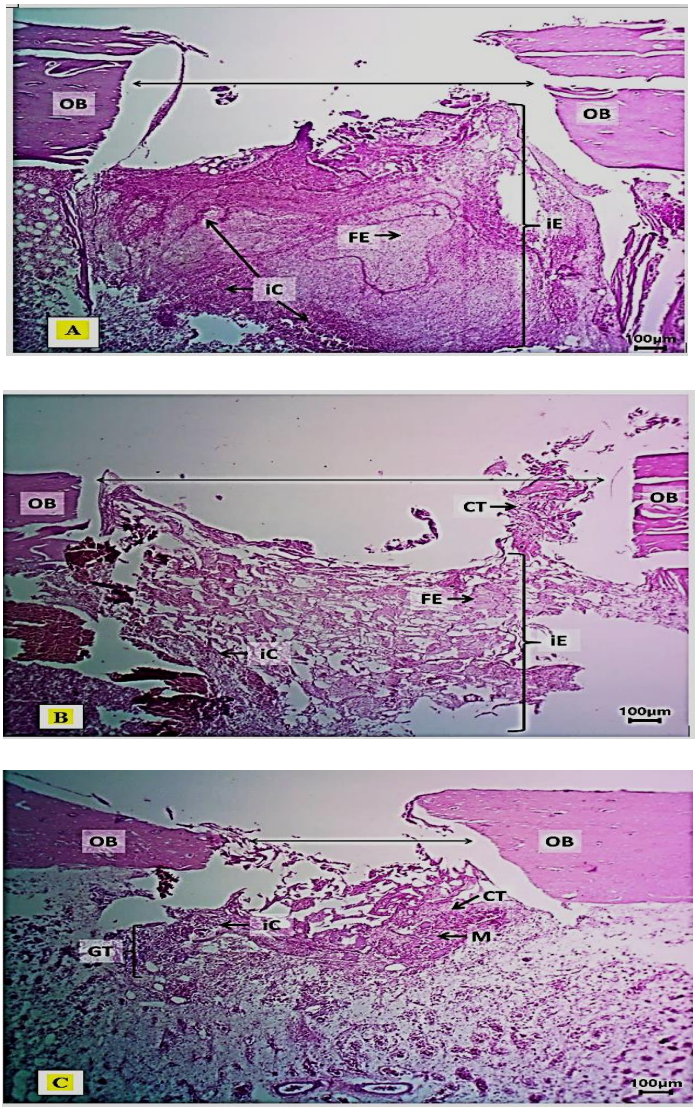

Figure (1): Three days period: section of rabbit's femoral bone, H&E stain. A: control negative group. B: control positive group. C: study group. Old bone (OB), inflammatory exudate (iE), including inflammatory cells (iC), fibrinous exudate (FE), connective tissue (CT), granulation tissue (GT), and the rest of the study material (M). 40X, Scale bar=100µm.

Seven days period

The histological section of the rabbit's femoral bone of the control negative group showed a wide defect area between the edges of old bone with granulation tissue formation, inflammatory cells infiltrate, and very few bone formations, while the control positive group showed a wide defect area between bone edges with granulation tissue formation, connective tissues, and inflammatory cells infiltrate with new bone formation. The study group showed a narrow defect bone site area between the edge of the old bone, under its granulation tissue, connective tissue with high new bone formation, cartilaginous tissue, and well angiogenesis (Figure 2).

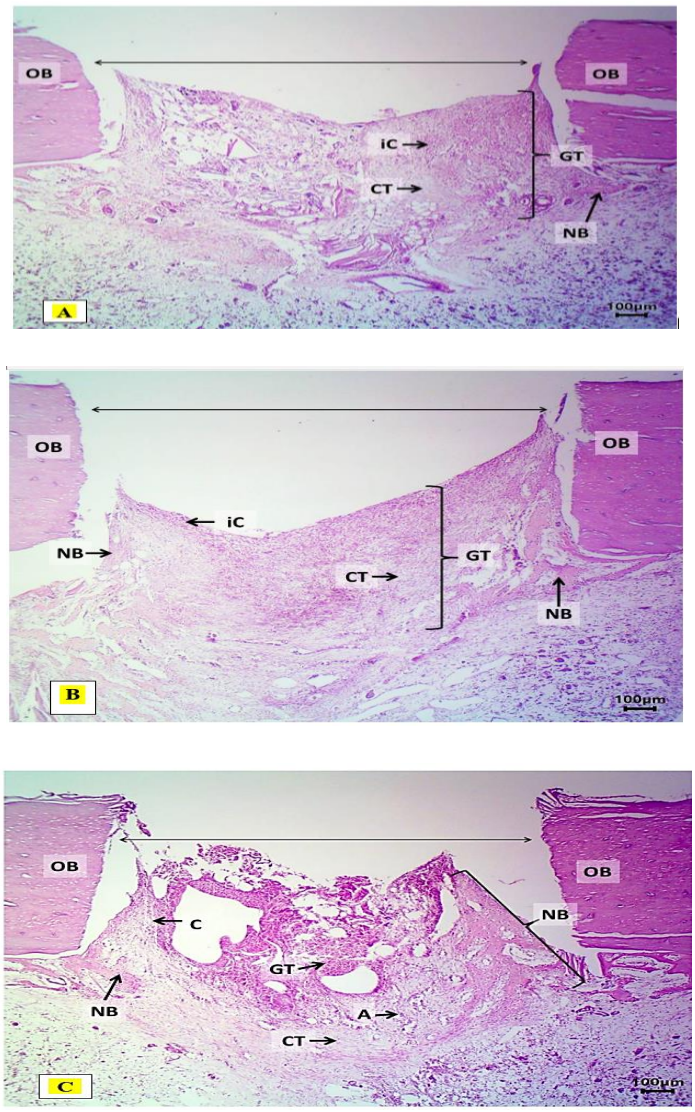

Figure (2): Seven days period: section of rabbit's femoral bone, H&E stain. A: control negative group. B: control positive group. C: study group. Old bone (OB), granulation tissue (GT), connective tissue (CT), inflammatory cells (iC), new bone formation (NB), cartilaginous tissue (C) and well angiogenesis (A). 40X, Scale bar=100µm.

Twenty -one days period:

The histological section of rabbit's femoral bone of the control negative group showed a wide defect bone site between the bone edges of the old bone, with new bone formation, cartilaginous tissue, connective tissue, and inflammatory cells infiltrate, while the control positive group showed a wide defect bone site area between the edges of the old bone, with new bone formation between it, cartilaginous tissue, and connective tissue. The study group showed partial occlusion of the defect bone site with highly new bone formation composed of highly osteoblasts, cartilaginous tissue, and the presence of the edge of the old bone (Figure 3).

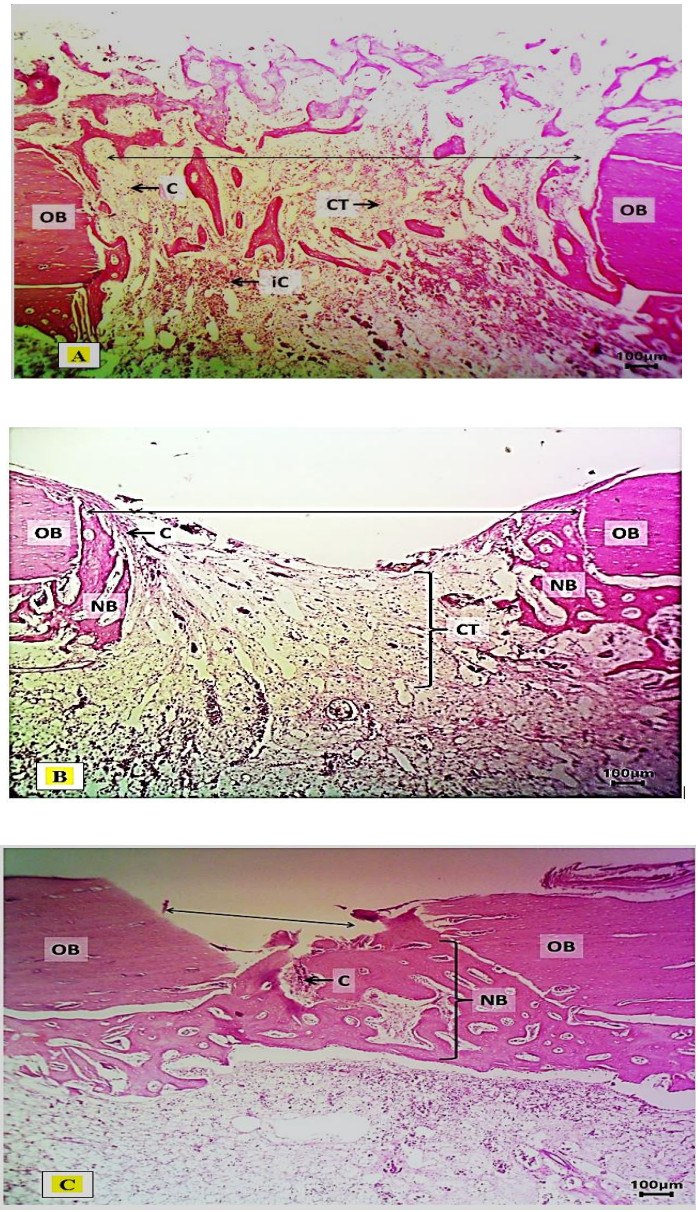

Figure (3): Twenty-one days period: section of rabbit's femoral bone, H&E stain. A: control negative group. B: control positive group. C: study group. Old bone (OB), new bone formation (NB), cartilaginous tissue (C), connective tissue (CT), and inflammatory cells (iC). 40X, Scale bar=100µm.

Twenty- eight days period:

The histological sections of the rabbit's femoral bone for the control negative group showed incomplete occlusion of the defect bone site area with islands of the new bone formation, cartilaginous tissue, and connective tissue with the presence of the edge of the old bone, while the control positive group showed incomplete occlusion of the defect bone site area with well-developed new bone formation, cartilaginous tissue, and connective tissue and presence of the edge of the old bone. The study group showed complete occlusion of the defect bone site area with a well-developed new bone formation composed of highly osteoblasts and the presence of the edge of the old bone (Figure 4).

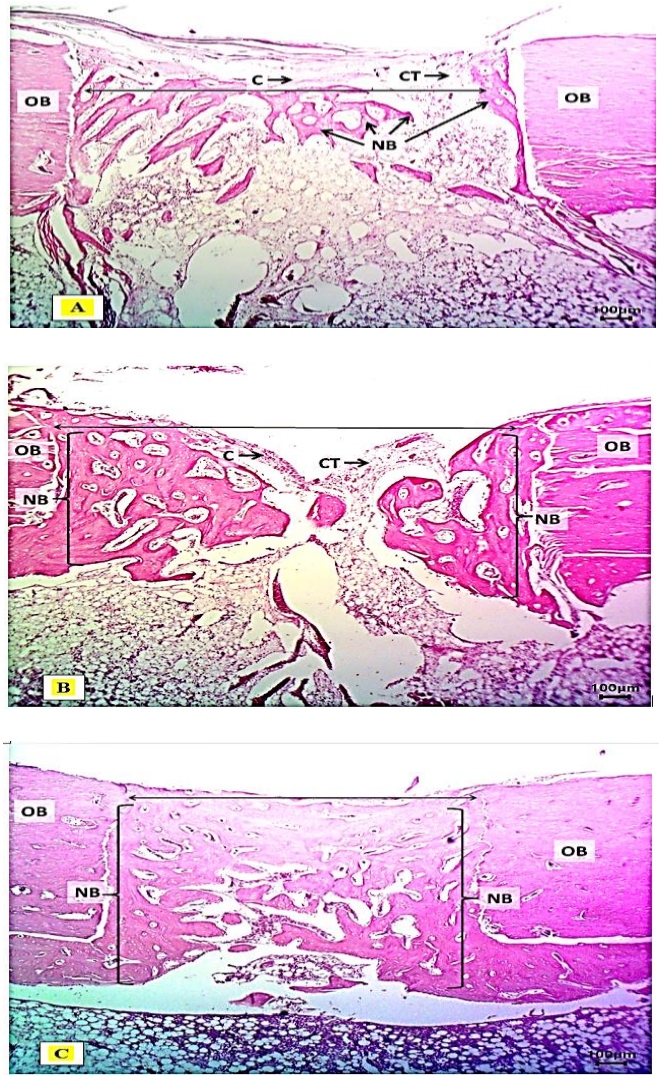

Figure (4): Twenty-eight day period: section of rabbit's femoral bone, H&E stain. A: control negative group. B: control positive group. C: study group. New bone formation (NB), cartilaginous tissue (C), connective tissue (CT) and presence at the edge of the old bone (OB). H&E stain, 40X, Scale bar=100 µm.

Table 2: Scores of the histological criteria for all groups at all periods

Periods / Groups	3 days	7 days	21 days	28 days	*p*-Value
Negative Control group	0.2±0.2 B b	0.8±0.2 B b	1.4±0.24 B a	1.8±0.2 B a	<0.001
Positive Control group	0.6±0.24 B c	0.8±0.2 B c	1.2±0.2 B b	2.2±0.2 A a	<0.001
Study material group	1.4±0.24 A b	1.6±0.24 A b	2.2±0.2 A a	2.8±0.2 A a	<0.001
***p*-Value**	**0.010**	**0.033**	**0.016**	**0.013**	

Data expressed as Mean ± SE (standard error) (N= 5 animals)
-different capital letters mean a significant difference among groups at $p \leq 0.05$.
-different small letters mean a significant difference among periods at $p \leq 0.05$.

DISCUSSION

Bone healing is a complex process that involves constant deposition, resorption, and remodeling (Santoso *et al.,* 2019). Various modalities have been recommended for the treatment of fractures; these can include physical, chemical, and biological modalities (Al-Mutheffer, 2014; Mafamane *et al.*, 2017).

After the application of estrogen hormone in incisional wounds, 3 days following skin incision, the control group had a defect area, no epithelium had formed, and necrotic tissue had formed (Al-Kadhimy and Ghani 2015). In contrast, the experimental group's histological observations at three days revealed an area of granulation tissue formation, epithelial cell migration, new hair follicle formation, numerous blood capillaries, and numerous inflammatory cells. This aligns with the findings of our study, where the HPC has a positive effect on bone healing by reducing inflammation during this stage of the healing process.

In vivo investigation of animal model bone-healing utility of growth hormone (GH), whether used as a powder to fill the dental socket or as an irrigation solution during implant placement. These applications have both increased implant stability and bone healing. Nevertheless, relatively little research examined the effect of GH therapy as locally administered bone regeneration materials to the extraoral region (cranio-facial), despite these positive results (Chaves *et al.,* 2020).

At 7 days of rat's tooth socket healing, after the tooth was extracted, it was shown that the socket treated with ethanol-containing Miswak extract gel was filled with immature fibrovascular granulation tissue (FVGT), rich in blood vessels. The bottom of the socket was filled with woven bone, and remnants of residual periodontal ligament were still adherent to the socket margin (AL Bayaty *et al.,* 2018). These results resemble our findings during this period, which showed high new bone formation, cartilaginous tissue and good angiogenesis of hole defects.

Al-Mutheffer (2014) found that using N. sativa oil extract as an epi-cutaneous therapy improves bone healing by promoting various processes of cell migration and differen-tiation, extracellular matrix formation, and organization towards calcification in different periods 1, 2, 3, 4, and 5 weeks. In the 3rd week, the treated group appeared to have a mature trabecular bone, fibrous connective tissue, and primary and secondary spongiosa, all encircled by active osteoblasts. The study group of our research showed new bone formation composed of highly osteoblasts between the edges of the old bone. This agrees with Naini *et al.*, 2024, who found InterOss® bone powder offered better bone reconstruction, less residual material, and a lower inflammation level, compared to Bone+B® during the healing period. Consequently, interposes® bone powder should be the superior choice for maxillofacial and facial surgery.

In the 4th and 5th weeks, the treated group's histological change was more evident, showing that compact bone with haversian canals filled the bone cavity more quickly than in the control group. The information presented here demonstrates the presence of numerous critical elements, including potassium, calcium, sodium, and other elements, which are abundant in black seed and crucial for bone formation and regeneration (Al-Mutheffer, 2014). The study group shows complete occlusion of the defect bone site with well-developed new bone formation composed of highly osteoblasts at the edge of the old bone.

Ali and Hamed (2023) found that through histological examination, the study assessed the accelerating effect of BCP (biphasic calcium phosphate) on bone formation when associated with I-PRF (injectable platelet-rich fibrin). Histological findings showed an increase in bone formation, which may be due to the increase in newly formed blood vessels, number of fibroblasts, and osteoid tissue formation in all BCP+i-PRF groups, and this was in the same line as our increased bone formation in the study group.

CONCLUSION

HPC illustrates the accumulation of mesenchymal stem cells that differentiated into osteoblasts, which facilitate and accelerate bone formation in the periods of the study.

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التاثير النسيجي لهرمون الهيدروكسي بروجستيرون كابرويت على التئام العظام بعد تطبيقيه على العيوب المستحدثة جراحيا

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العظام عبارة عن نسيج ضام حي مشبع بالأوعية الدموية، وهو مسئول عن الوظائف الايضية والميكانيكية الهامة. تم عزل البروجستيرون لاول مرة عام ١٩٣٤ من قبل مجموعات تدرس وظيفة الغدد الصماء في الجسم. تم اختيار ٦٠ ارنبا منزليا ذكرا بالغا، متوسط وزنها ١٧٥٠ غم، واعمارها ما بين ٨-١٠ اشهر. بعد تخدير الارنب، تم اجراء شق بطول ٤ سم في الساق اليسرى فوق عظم الفخذ، وازاحة العضلات لكشف عظم الفخذ. تم عمل فتحتين بسنفرة مستديرة ٣ ملم، والمسافة بين الفتحات حوالي ١ سم. في المجموعة الضابطة السلبية تركت الفتحتين دون ملئهما باي مادة، بينما في المجموعة الضابطة الايجابية تم ملئ الفتحتين بوضع قطعة من الجلفوم، اما مجموعة الدراسة، فتم ملىء الفتحتين بواسطة الجلفوم المنقوع في الهيدروكسي بروجستيرون كابرويت. أظهرت النتائج في مجموعة الدراسة غلق الفتحتين بشكل أسرع من المجموعتين الاخريين، في فترة السبعة ايام اظهرت منطقة موقع الفتحتين، ضيقة بين حافة العظم القديم، وتحتها نسيج حُبِيّبِي (granulation)، ونسيج ضام مع تكوين عظمي جديد مرتفع، ونسيج غضروفي، وتولد الاوعية بشكل جيد. مع القيمة p-value 0.033. وخلصت الدراسة الى ان تقنية الهيدروكسي بروجستيرون كابرويت عملت على تسريع عملية شفاء العظام في الفترات الزمنية المختلفة عن طريق تنشيط الخلايا العظمية لتكوين العظام.