Spring 1981

The Effects Of Osteum On Fracture Healing In Mice

Joseph Russell
Carroll College

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THE EFFECTS OF OSTEUM™ ON FRACTURE HEALING IN MICE

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

Joseph Warren Russell
March 24, 1981
This thesis for honors recognition has been approved for the Department of Biology.

Dr. James J. Manion, Advisor

Rev. Joseph D. Harrington

Henry E. Burgess

March 24, 1981
ACKNOWLEDGEMENTS

I wish to thank my advisor, Dr. James Manion, and my readers, Fr. Harrington and Mr. Burgess, for their time and effort. I would also like to thank Dr. William J. Hadlow and Monty Thompson of the Rocky Mountain Laboratory, Hamilton, Montana for their help in preparing the slides for this investigation.
Osteum (Schering Corporation - sodium oleate solution) was assessed for its effects on osteoblast proliferation and/or activity. Mice given 0.1 cc of sodium oleate solution near site of fracture were tested against controls. Qualitative and quantitative results show that Osteum accelerates osteoblast proliferation and/or activity, but mechanism is unclear.
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INTRODUCTION

Investigators have been concerned with enhancement of fracture healing for many years. These people have come up with a few substances that seem to have some beneficial results but only affect the mineral phase. In recent years investigators have been looking for substances that might affect some initial phase in osteogenesis. Schering has come up with a drug that seems to affect osteoblastic proliferation (10). Mice, with their rapid regeneration time and ease of tissue sectioning, were used to investigate this phenomenon.
Osteoblasts

Osteoblasts have long been associated with formation of bone. During active growth of bone they appear in a continuous layer. These cells are frequently connected with one another by thin cytoplasmic processes (2). Osteoblasts, in an active state, appear to be cuboidal in shape with a breadth of 15 to 20 microns (5).

Osteoblasts appear to come from the same stem cell as do osteocytes (associated with the maintenance of bone as a living tissue) and osteoclasts (associated with the resorption of bone). During active growth, whether reconstructive or formative, there can be noticed frequent transformations occurring from one to another of these morphologically different forms. These cells retain their potencies common to all three forms. The morphology of these stem cells at any given time depends upon the function it is being called upon to perform (3, 5). In an inactive state the osteoblast cell takes on a spindle shape and will resemble fibroblast cells (6). Osteoblasts, whether in an active or an inactive state, are the major components of the inner layer of the periosteum (1), sometimes called the cambium layer, and the

2
endosteum, which is a condensation of the stroma of bone marrow.

Osteoblasts play an active part in osteogenesis by forming collagenous fibers and depositing ground substance. In a stage of active osteogenesis the osteoblasts contain an abundant rough-surfaced endoplasmic reticulum and a prominent Golgi complex next to the nucleus. They are also observed to contain glycogen, vesicles, and a large amount of mitochondria. These mitochondria often contain electron-dense granules associated with their cristae. Pinocytic vesicles are readily observed at the plasma membrane in contact with the osteoid (the organic matrix covering the surface of bone) (2, 4).

Osteoblasts, in their active state, have been observed to have a marked increase in their uptake of precursor amino acids used in the synthesis of protocollagen. The most abundant amino acids taken up for synthesis are glycine and proline. Amino acid uptake by osteoblasts in rat calvaria occurs by two different mechanisms. These mechanisms are sodium-dependent and sodium-independent. The sodium-dependent mechanism is responsible for the uptake of imino acids, one being proline. Neutral amino acid uptake in osteoblasts occurs by both mechanisms cited. Under some circumstances it has been noted that the rate-determining factor in collagen synthesis is found to lie within the uptake of proline (9). It has been shown that most of the protein secreted from this cell is in the form of collagen (3, 9).
The deposition of hydroxyapatite is found to lie within the granules in the mitochondria of the osteoblast. Osteoblasts in active osteogenesis are characterized by electron-dense cytoplasm (4, 6, 9). It has been shown that this electron density has been correlated with the amount of mineral granules found in the inner membrane of mitochondria (4). Hydroxyapatite is the major inorganic constituent of bone matrix.

As pointed out by Parfitt, enhanced synthesis of new bone is in direct correlation with an increase in osteoblast number (9).

Enhancement of Osteogenesis

In recent years investigators have been concerned with the enhancement of fracture healing and some of its mechanisms. Some vitamins, minerals, and hormones have been tested with good results.

The vitamins most extensively studied have been C and D. Intensive therapy with vitamin C and calcium have been shown to have beneficial effects (11). Vitamin D has been noted to have an effect on calcium uptake by the osteoblasts (6, 7). Matthews has demonstrated the dependency of mitochondrial granule density to vitamin D (6). These vitamins primarily affect only the mineral phase of fracture repair.

Calcium levels have an effect on the rate at which osteogenesis proceeds. Increased intracellular calcium levels, as noted by Talmage (cited by Mills), is believed
the stimulus which activates RNA synthesis. This increases the rate of maturation of undifferentiated bone cells to osteoblasts (7). Sodium, as stated earlier, plays an important part in the uptake of some precursor amino acids needed for the synthesis of protocollagen.

Hormones such as growth hormone, thyroxin, and anabolic hormone from the testes have an effect on speeding up of fracture healing. The parathyroid hormone is effective in the stimulation of intracellular calcium influx (9, 11).

Udupa has isolated an unsaturated ketosteroid from an indigenous herb *Cissus quadrangularis* which has accelerated fracture healing. The mechanism of accelerated fracture healing seems to lie in the steroid's ability to produce a positive nitrogen and calcium balance. This steroid also seems to have an indirect stimulative effect on connective tissue cells especially the fibroblasts and osteoblasts (11).

Studies performed at the United States Army Institute of Dental Research have shown that two copolymers, polylactic acid and polyglycolic acid, show good tissue compatibility and aid in the repair of certain fractures. These compounds, when administered at the site of fracture, lay down a matrix where uniform proliferation of fibroblasts and osteoblasts occurs (8).

Osteum

In 1971 the Schering Corporation came up with a "chemo-therapeutic agent that accelerates healing and reduces costly recovery time." The agent is Osteum and is a sodium oleate
solution. The chemical makeup of the drug is as follows:

- Sodium oleate 50 mg/ml
- Sodium phosphate 1 mg/ml
- Alcohol 1.5%
- Sodium hydroxide to adjust pH

This drug has been used exclusively on horses to aid in the healing of splints, microfractures and fractures of the cannon bone (10).

Studies by Wisner show that use of this drug is over 90% successful in accelerating bone healing (12).

In early 1976, Schering stated that Osteum exerts its activity by stimulating infiltration of cellular blood components which subsequently differentiate into fibrous and/or connective tissue (10). Cooper (stated in Schering) reported that his studies demonstrated an increase in osteoblastic activity yielding a more rapid formation of a callus than in control fractures (10). Later in 1976, Wisner stated that the means by which Osteum achieves its therapeutic effect has not been defined. Some theories on how the drug works are: the increase in a specific lipid front, adjustment of the ionic balance, and adjustment of the pH at the fracture site. Undifferentiated bone cell maturation is another plausible theory of how Osteum exerts its effect (12).
MATERIALS AND METHODS

Twenty-seven mice ranging from five to seven months were obtained from McLaughlin Research Center in Great Falls, Montana. These mice were kept in groups of three, in cages containing ample sawdust bedding, before and during experimentation. The diet of the mice consisted of Purina Rat Chow (containing sodium and calcium not more than 3% by volume) and water given ad libitum.

Experimental Procedure

Before attempting breakage of the right tibia and fibula, mice were anesthetized using ether in an anesthetic jar. Mice were monitored closely while in this jar. When mice had reached a state of deep rhythmic breathing and total limpness of appendages, they were immediately removed and the break made. The break was made by placing the mouse on its back, putting the right leg (medial side up) over a one-milliliter pipette, and applying equal pressure down at the knee and ankle with the thumbs. The break made was in the form of a complete fracture. These fractures were allowed to heal without any splintage. Mice used as controls were watched until fully recovered, then returned to their cages. Immediately after the break was performed, experimental mice were injected with a 0.1 cc dose of sodium oleate.
The injection was given in the right thigh with a 26-gauge needle and 1.0-ml disposable Plastipak syringe. These mice were also watched through recovery and returned to their cages.

After letting the bone regenerate for four to five days, mice were sacrificed in the euthanasia cage using CO$_2$ and subsequently placed in a freezer. After duration of 24 hours in the freezer, mice were removed and the right leg of each was detached and skinned. Each detached leg was then placed in a neutral buffered 10% formalin solution for preservation. Preserved legs were trimmed of some muscle on their lateral surface. Trimmed legs were subsequently placed in 5% formic acid for decalcification. The decalcification process was allowed to run for 28 hours. Following decalcification the tissue was washed in tap water for 16 hours to remove acid. In preparation for tissue processing, the tissue sample was placed in tissue cassettes and soaked in 70% alcohol for 24 hours. Following initial dehydration the tissue cassettes were placed into an Autotechnicon Tissue Processor. The process is as follows:

1. Graded washings in alcohol to dehydrate the tissue. Dehydration is carried out by a series of six alcoholic washings running from 70% to 100% alcohol.

2. One washing in Toluene to clear alcohol (alcohol is not miscible with paraffin).

3. Infiltration of the tissue by paraffin. This is obtained by three changes in paraffin kept at 59°C.
The next step in the sectioning procedure was to imbed paraffin-infiltrated tissue in a block of paraffin. This was accomplished with the use of a Tissue-Tex II tissue imbedding center. Once blocks were obtained they were allowed to cool for several hours before attempts were made to section.

Sectioning of the tissue sample was done on a Lietz Rotary Microtome. The microtome was set to cut at 5 microns. Longitudinal sections were made of the bone.

Sections were mounted on glass slides and placed in a 60°C oven for 30 minutes. After sections were fixed to the glass surface, two sample slides were used to determine an appropriate staining procedure that would best facilitate observation of osteoblast and osteoid.

Staining Procedures

The staining procedures used were the Hematoxylin and eosin (H and E) and the Periodic acid-Schiffs (PAS) and are as follows:

H and E routine.

1. Wash in 2 changes of Xylene at 3 minutes each to clear paraffin from the tissue.
2. Place in absolute alcohol for 2 minutes.
3. Place in 95% alcohol for 2 minutes.
4. Run under tap water long enough to clear the alcohol, hence rehydrating the tissue.
5. Place in Harris' Hematoxylin for 5 minutes.
6. Run under tap water to remove excess stain.
7. Differentiate tissue in acid alcohol (1% HCl in 70% alcohol) for a few seconds.

8. Rinse in tap water.

9. Place tissue in saturated lithium carbonate for 15 seconds to neutralize acid.

10. Rinse again in tap water for 5 minutes.

11. Place in 95% alcohol for 2 minutes.

12. Place in acidified eosin for 30-40 seconds.

13. Rinse again in 95% alcohol.

14. Place in absolute alcohol for 2-3 minutes to remove any water.

15. Place in a 30-50 acetone and xylene mixture for 3 minutes.

16. Make 2 washings in xylene at 3 minutes each.

17. Coverslip slide using permount.

PAS technique.

1. Wash in 2 changes of xylene at 3 minutes each to clear paraffin from the tissue.

2. Place in absolute alcohol for 2 minutes.

3. Place in 95% alcohol for 2 minutes.

4. Run under tap water long enough to clear the alcohol, hence rehydrating the tissue.

5. Place in 0.5% Periodic acid for 8-10 minutes.

6. Rinse well in distilled water.

7. Place next in Schiff's stain for 20 minutes. Schiff's reagent must be kept in cold dark storage.

8. Wash in running tap water for 15-20 minutes to wash away acid.

9. Place in Harris' hematoxylin for 5 minutes.

10. Rinse in tap water for 2 minutes.
11. Differentiate in acid alcohol (1% HCl in 70% alcohol) for a few seconds.

12. Rinse again in tap water.

13. Neutralize in saturated lithium carbonate for 15 seconds.

14. Wash for 5 minutes in running tap water.

15. Place in saturated tartrazene in cellosolve (2 ethoxy-ethanol) for 4-5 minutes.

16. Wash in 95% alcohol.

17. Dehydrate in absolute alcohol for 2 minutes.

18. Clear in xylene for 1 minute.


Prepared slides were now ready for observation. Qualitative and quantitative data was obtained by inspection using an American Optical microscope at 100 power.
RESULTS

Of the 27 mice, results were obtained on 18. No data on 9 of the mice was due to poor breaking procedure on one and accidental disposal of 8 by the janitorial service of Carroll College. Numerical titles have been given to these mice and are as follows:

1a. Injected allowing bone to regenerate 5 days
1b. Injected allowing bone to regenerate 5 days
2a. Injected allowing bone to regenerate 4 days
2b. Injected allowing bone to regenerate 4 days
3a. Control allowing bone to regenerate 4 days
3b. Control allowing bone to regenerate 4 days
4a. Control allowing bone to regenerate 5 days
4b. Control allowing bone to regenerate 5 days
5. Control allowing bone to regenerate 4 days
6. Injected allowing bone to regenerate 4 days
7. Injected allowing bone to regenerate 4 days
8. Control allowing bone to regenerate 4 days
9. Control allowing bone to regenerate 5 days
10a. Injected allowing bone to regenerate 5 days
10b. Injected allowing bone to regenerate 5 days
11a. Injected allowing bone to regenerate 5 days
11b. Injected allowing bone to regenerate 5 days
12. Control allowing bone to regenerate 5 days
Different numerical titles for the same procedure designate different testing times.

All breaks to be described are from tibial observations except where noted. Qualitative and quantitative data taken on these fractures are as follows:

1a. Osteoblast activity was good with cambium layer 100-130 microns deep. There was much osteoid material found within this layer. There was good migration of fibroblast into marrow space. No multinucleated osteoclasts were noted.

1b. Good osteoblastic activity noted with cambium layer 100-160 microns deep. There was a moderate amount of osteoid material present within. Good migration of fibroblast was noted. New osteocytes were noted on the periphery of the dense bone.

2a. Osteoblast activity was good. The cambium layer was 100-150 microns deep with much osteoid material present. Noted good migration of fibroblast cells. No osteoclastic activity present. Newly incorporated osteocytes were noted.

2b. Osteoblast activity was good. The cambium layer ranged from 100-200 microns in some places with moderate amount of osteoid material present. New osteocytes were noted.

3a. Poorly sectioned. Proximal portion of fracture barely in section. Noted fair osteoblastic activity with good migration of fibroblasts. Cambium layer ranged from 40-80 microns with a trace amount of osteoid present.
3b. Good osteoblastic activity noted around spicules lying in soft tissue. Cambium layer ranged from 80-120 microns with some osteoid material present. No osteoclasts or new osteocytes were noted. Good migration of fibroblasts noted.

4a. Osteoblast activity was moderate with depth on cambium layer being 60-100 microns. Spicules noted with some amount of activity. Some new osteoid material was noted with no osteoclasts observed. Fibroblast migration was good.

4b. Osteoblast activity good with cambium layer having a depth of 80-140 microns. Some osteoid material was present. Good migration of fibroblasts noted.

5. Poorly sectioned. Minimal osteoblastic activity was noted with cambium layer 40-60 microns in depth. Trace amount of osteoid material present. Moderate to good fibroblast migration noted.

6. Poorly sectioned. Moderate osteoblastic activity was noted. Cambium layer ranged from 60-120 microns with good osteoid material found within. Good migration of fibroblasts noted.

7. Sporadic osteoblast activity with cambium layer depths from 80-120 microns along a fibula fracture. Tibial fracture has moderate osteoblastic activity. Moderate osteoid material found within cambium layers near both fractures.
8. Moderate osteoblast activity was noted with cambium layer depths of 60-80 microns. Migration of fibroblast was good. No new osteocytes were found. Moderate amount of osteoid material observed.

9. Good osteoblastic activity was found with cambium layer depth ranging from 80-100 microns. Some osteoid material was present. Good migration of fibroblast cells was noted.

10a. Poorly sectioned. Good osteoblast activity noted where fracture was in section. Good migration of fibroblasts was observed. Good amount of osteoid material present. No quantitative data could be obtained.

10b. Good osteoblast activity was observed throughout cambium layer. Cambium layer ranged from 100-140 microns. Much osteoid material was found throughout cambium layer. Newly incorporated osteocytes were also noted. Good migration of fibroblasts noted.

11a. Poorly sectioned. Moderate osteoblast activity was noted. Good migration of fibroblasts was observed. No quantitative data could be obtained. Moderate amount of osteoid material present.

11b. Fibula fracture shows good osteoblast activity with good amounts of osteoid material present within cambium layer. Cambium layer ranged from 80-120 microns in depth. Good migration of fibroblast cell was noted.
12. Poorly sectioned. Migration of fibroblast was good but very little differentiation to osteoblast cells was noted. No quantitative data could be obtained. Sections show trace of new osteoid material.
TABLE 1. TABLE OF QUALITATIVE DATA

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Plate 1. Injected leg allowing bone to regenerate 4 days (long. section)

Plate 2. Control leg allowing bone to regenerate 4 days (long. section)
Plate 3. Injected leg allowing bone to regenerate 5 days (long. section)

Plate 4. Control leg allowing bone to regenerate 5 days (long. section)
Statistical Analysis of Quantitative Data

Cambium layer low range values:

Injected - 4 days  mean = \( \bar{m} = \frac{\sum fx}{n} \)
\[ \bar{m} = \frac{100 + 100 + 60 + 80}{4} \]
\[ \bar{m} = 85.0 \text{ microns} \]

standard deviation = \( s = \sqrt{\frac{\sum (f_{\text{variants}})^2}{n}} \)
\[ s = 19.15 \]

standard error = \( se = \frac{s}{\sqrt{n}} \)
\[ se = 9.58 \]

Injected - 5 days  mean = \( \bar{m} = 95.0 \text{ microns} \)

standard deviation = \( s = 10.0 \)

standard error = \( se = 5.0 \)

Control - 4 days  mean = \( \bar{m} = 55.0 \text{ microns} \)

standard deviation = \( s = 19.15 \)

standard error = \( se = 9.58 \)

Control - 5 days  mean = \( \bar{m} = 73.3 \text{ microns} \)

standard deviation = \( s = 11.5 \)

standard error = \( se = 6.6 \)

Cambium layer high range values:

Injected - 4 days  mean = \( \bar{m} = 147.5 \text{ microns} \)

standard deviation = \( s = 37.75 \)

standard error = \( se = 18.87 \)

Injected - 5 days  mean = \( \bar{m} = 137.5 \text{ microns} \)

standard deviation = \( s = 17.02 \)

standard error = 8.5
Control - 4 days  mean = $\bar{m} = 85.0$ microns
standard deviation = $s = 25.2$
standard error = $se = 12.6$

Control - 5 days  mean = $\bar{m} = 113.3$ microns
standard deviation = $s = 23.1$
standard error = $se = 13.3$

Comparison of Data Using Student T

t = \frac{\bar{m}_1 - \bar{m}_2}{\sqrt{(se_1)^2 + (se_2)^2}}

Injected and control at 4 days:

Low range value  $t = \frac{85.0 - 55.0}{\sqrt{(9.58)^2 + (9.58)^2}}$

$\begin{align*}
t &= 2.2
\end{align*}$

High range value  $t = \frac{147.5 - 85.0}{\sqrt{(18.87)^2 + (12.6)^2}}$

$\begin{align*}
t &= 2.75
\end{align*}$

Injected and control at 5 days:

Low range value  $t = \frac{95.0 - 73.3}{\sqrt{(5)^2 + (6.6)^2}}$

$\begin{align*}
t &= 2.6
\end{align*}$

High range value  $t = \frac{137.5 - 113.3}{\sqrt{(8.5)^2 + (13.3)^2}}$

$\begin{align*}
t &= 1.53
\end{align*}$

Statistical Data

degrees of freedom = 3

$\begin{align*}
t_{.1} &= 1.638 \\
t_{.05} &= 2.353 \\
t_{.025} &= 3.182
\end{align*}$
DISCUSSION AND CONCLUSIONS

Longitudinal sections of the bone were made to facilitate observation of the fracture. The irregularity of the breakage sites along the tibia made it quite difficult to obtain cross sections through the break. Longitudinal sections also made it possible to quantitate cambium layer depth. Cambium layer depths were taken at sites where active osteoblasts were found. The Hematoxylin and eosin staining procedure was used instead of the Periodic acid - Schiff's procedure as it provided more definable results.

Gross observation of fracture sites seemed to show a difference in osteoblast number and their activity in injected and control groups. Osteoblast number seemed to be greater in the injected groups when compared to controls. Observing the amount of osteoid material present in the cambium layer also pointed to injected groups being more active in osteogenesis. Since osteoid is a product of the osteoblast cell, this points to enhanced (or greater) osteoblast activity. Increased amounts of osteoid material when compared to controls could be due to faster proliferation and/or maturation of undifferentiated peripheral bone cells to osteoblast cells. Migration of fibroblast cells seems to be independent of the effects
of the Osteum. Migration of fibroblasts was similar in both injected and control groups.

Statistical analysis of cambium layer depth shows the same results. In comparing the low range values of cambium layer depth in experimental and controls at 4 days, the mean depth is greater in experimental when compared to controls at a 90% confidence level. In comparison of high range values at 4 days, there is a 95% confidence that mean experimental cambium layer depth is higher than in controls. At a 95% confidence level, low range values of experimental were significantly higher than in controls allowed to regenerate for 5 days. When comparing high range values for experimental and controls at 5 days, the confidence level drops slightly below 90% in comparison by Student t.

Even though these results reflect enhanced osteoblast formation and/or activity, the mechanism of this phenomenon is still unclear. It is my feeling that enhanced osteoblastic activity is a secondary response. I have come to this conclusion by observation of fibroblast migration. Since migration of fibroblasts seems to be constant between experimental and control mice, I feel that this drug does not exert its effect directly on undifferentiated bone cells. Wisner postulated that Osteum might have an effect on increasing the specific lipid front at the fracture site (12). Udupa, working with an unrelated lipid, has found that his substance affects enhancement by producing a positive nitrogen and calcium balance (11). If a mechanism such as Udupa's
occurred with Osteum, I feel that a more rapid formation of mature osteoblasts would take place. More investigation of this drug is needed. When more is known about the mechanism(s) involved here, human application of this drug might evolve.


