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Effect of prolonged administration of caffeine during fracture repair progression in rats

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Abstract
Bone fracture is a complete or partial breakage of the bone structure. Fracture repair is considered a regenerative process whereby healing occurs through the formation of new bone tissue indiscernible from uninjured bone. Fracture repair is the process of reducing and realigning fractured bone ends together in other to achieve healing. Caffeine, or 1, 3, 7-trimethylxanthine, is an alkaloid present in more than one hundred plant species. It is used as stimulants and it affects calcium mobilization in the body. It may predispose to fracture.
Methodology; a total of 30 rats procured from a known breeder (University of Maiduguri quarters) and acclimatized for 14 days and divided into 2 group A (15 rats), and group B (15 rats). Group A were administered caffeine orally at 500 mg/kg while group B were given placebo which served as control. Samples from fractured tibiae were harvested on day 14, 28, and 42 respectively. The samples were decalcified using formic acid and stained with H and E (Hematoxylin and Eosin) and viewed under microscope at X10, X40 and X100 magnifications. The results showed no significant difference between the treated and the control group on day 14. However, we observed histological difference on day 28 and 42 even though there was no statistical difference using student's t test. (p>0.05) between the caffeine treated group and the control group.
The finding shows that caffeine intake at 5mg/kg for more than 14 days and above post fracture may delay fracture repair.

Keywords: Fracture, caffeine, rat, tibia, histology

Introduction
Fracture repair is a dynamic and complex process that involves a programmed interplay of cells, some growth factors and components of the extracellular matrix of a bone to form a new bone bridging a lost bony architecture. Fracture repair has been conventionally categorized into four main stages (hematoma formation, soft callus, hard callus, remodeling) where each stage is associated with specific defined cellular processes. (Bolander *et al*., 1992) [3]. Fracture repair is aimed at restoring the function and integrity of the affected bone. Several techniques have over the years been developed and employed to facilitate fracture repair processes which are aimed at achieving reduction, immobilization and application of braces such as splints, casts, internal and external skeletal fixatives and other advanced surgical management procedures. (Mana *et al*., 2022) [9]. The choice of approach to fracture management usually depends on certain factors that include but not limited to bone type, location, type of fracture (Open, close, simple, compound, linear, oblique, transverse or comminuted), and severity. Other factors include patient age and health status. Fracture healing in general is aimed at achieving a faster resolution of the damaged bony architecture to enable the patient return to normalcy. The four-stage delineated stages of fracture repair progression emanates from histological observations of healing processes in animal models and human patients. However, decades of research work has explored both the cellular and molecular events that drive the underlying processes (AI-Aql *et al., 2*008) [1]. Histologically, inflammatory cells, vascular cells, osteochondral progenitors, and osteoclasts are key players in the reparative process of fracture healing. Fracture repair at the molecular stage is driven by three major classes of factors which include pro-inflammatory cytokines and growth factors, pro-osteogenic factors, and angiogenic factors (Barnes *et al.*, 1999) [2].

These factors are responsible for laying the relevant morphogenetic fields by recruiting and assembly of cells, stimulating growth and/or differentiation. The formation of a hard callus is preceded by gradual depletion of soft callus and the formation of new vessels for blood supply. The new hard callus formed is typically irregular and less-remodeled. Research has showed that formation of hard callus can occur without a cartilaginous template in demonstrated in intramembranous bone formation (Peak mechanical stability) or where bone formation occurs close to an adjacent mineralized structure (Appositional bone growth) (Barnes, 1999) [2]. The temporarily laid woven bone architecture is a protein derived and mineralized extracellular matrix complex. Matured osteoblasts are responsible for its synthesis, which subsequently is been differentiated from osteoprogenitor cells stimulated by osteogenic factors. The BMP family members are the main intermediaries of this process and over the years proven in abundance for *de novo* bone formation (Chen *et al*., 2007) [4].

Additional mediators have been expressed at this stage; however, it remains vague if their impact on bone healing majorly impacts osteogenic differentiation against osteoprogenitor migration/proliferation (Gerstenfeld *et al*., 2003) [5]. The origin of osteoprogenitor cells involved in fracture repair is still unclear. Periosteum has been discovered to be a rich hub for the synthesis of osteoblast hence fracture repair is often been impeded when it is damaged or disrupted (Malizos *et al*., 2006) [8]. Remodeling as the terminal stage of bone healing involves the transformation of woven hard callus to a cortical bone which is also termed as secondary bone formation (Gerstenfeld *et al*., 2003) [5]. Originally, this transformation comprise of phasing the woven bone into lamellar bone, however the originating cortical layout is eventually restored. This remodeling process is driven by a coupled process of orderly bone resorption followed by the formation of lamellar bone (Gerstenfeld *et al*., 2003) [5]. The process of neo-vascularization is spared. Thereafter, the damaged soft tissues are repaired and the fracture site is covered by soft callus and later hard callus. The bridging hard callus is eventually remodeled to re-establish the original geometry and function of the damaged tissue. In instances where there is absolute stability, or in a reduced metaphyseal fractures even without the appearance of an intermediate cartilaginous soft callus, formation of intramembranous hard callus dominates (Schindeler *et al.*, 2008) [11].

Caffeine, or 1, 3, 7-trimethylxanthine, is an alkaloid present in more than one hundred plant species. It is a stimulant that is present in various beverages and foods, including coffee, tea, chocolate, and some medications. It is well known for its ability to increase alertness and improve mental performance, but it can also have a number of other effects on the body. Studies have shown that the caffeine intake may lead to calcium loss in the urine in humans (Kynast *et al.*, 1994) [6]. There have been several studies that have examined the effect of caffeine on fracture repair in animals. These studies have generally found that caffeine can delay or impair the healing process of bones (Macedo *et al.*, 2015) [7]. Furthermore, Moreno *et al.*, (2022) [10] explained that most research conducted in rats found that caffeine intake was associated with a decrease in bone density and a delay in the healing of fractures and also found out that caffeine consumption reduced the strength of bones in rats and impaired the formation of new bone tissue.

Methodology

A total of 30 rats procured from a known breeder (University of Maiduguri quarters) and acclimatized for 21 days and divided into 2 groups; Group A=15 rats and Group B=15 rats. Group A were given caffeine orally (5mg/kg) whereas group B served as control. Samples from fractured tibiae were harvested on day 14, 28, and 42 respectively. The samples were decalcified using formic acid and cross sections were made following standard histological techniques and stained with H and E (Hematoxylin and Eosin) and viewed under microscope at X10, X40 and X100 magnifications.

Statistical analysis

All data obtained were expressed as mean ± standard deviation and analyzed using two tailed T test, within the groups (Instate Version 3). P-value < 0.05 was considered statistically significant.

Results

On day 14 post surgery, the cellular picture of the fracture was characterized by sparsely distributed collagen with distinct fibroblast (black arrow), marked osteoblasts (blue arrow) and few chondrocytes (Pink arrow) with marked cellular infiltration (Figure 1 A). However, in Figure 1B, it reveals infiltration of fibroblasts and mononuclear cells from the periosteal sheath (back arrow) with scanty admixture of collagen and presence of empty space. Furthermore, debris and hematoma formation around the fracture site was observed in the rats for all the groups at day 14 in this study **(**Figure 1). This indicates that osteotomy of the tibia created injury resulting in blood vessel disruption hence the hematoma and debris into the fracture site. There were no significant variations in the histological sections on day 14 for caffeine treated and control group.

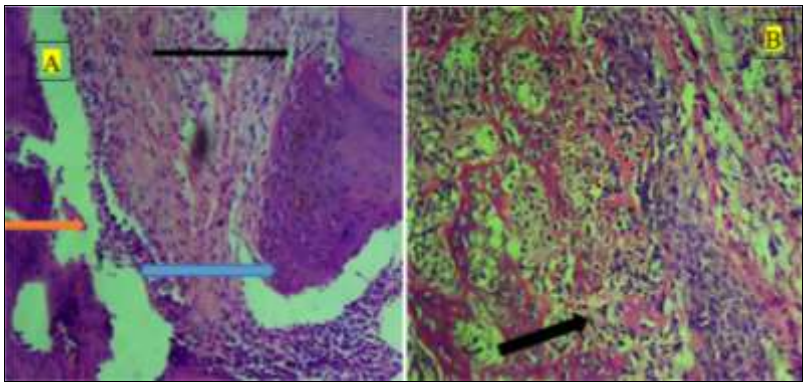

Fig 1: A) Caffeine Treated group: Zone of fracture showing infiltration of fibroblast and mononuclear cells from the periosteal sheath (back arrow), scanty admixture of collagen and presence of empty space. B) Control Group: Moderate collagen formation, presence of mononuclear cells (black arrow), chondrocytes formation (blue arrow) at the periphery

Furthermore, on day 28, we noticed a zone of fracture with fewer collagen deposition and marked angiogenesis (black circle) in the caffeine group. We also observed some few mononuclear cells at the periphery (blue arrow) and presence of fibroblast (black arrow) Figure 2A. This signifies a delay fracture repair. However, in the control group, we notice numerous mononuclear cells (black arrow) with collagen deposition and presence of osteocytes (Figure 2B).

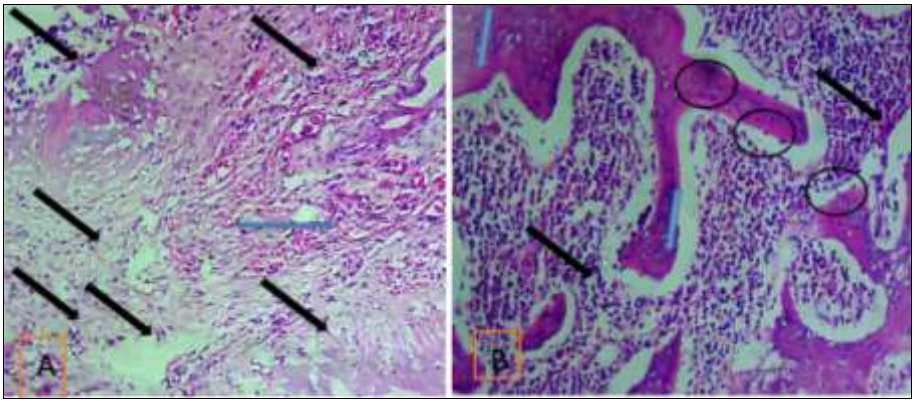

Fig 2: A) Caffeine Treated group: Zone of fracture showing few collagens deposition, marked angiogenesis (black circle), few mononuclear cells at the periphery (blue arrow) and presence of fibroblast (black arrow) B) Control group: Numerous mononuclear cells (black arrow) with collagen deposition and presence of osteocytes (black arrow)

On day 48, in the caffeine group was observed to predominantly demonstrate early stage osteoid callus and neither mineralization nor well-formed medullary cavity was seen. This signify significant delay in fracture repair progression. In the Caffeine treated group, the cellular picture demonstrated hypertrophic chondrocyte with the presence of collagen and scanty lamellar bone deposition (Blue arrow), diffused angiogenesis and presence of chondrocytes (black circle) and mononuclear cells (Figure 3B). This shows a moderate fracture repair progression.

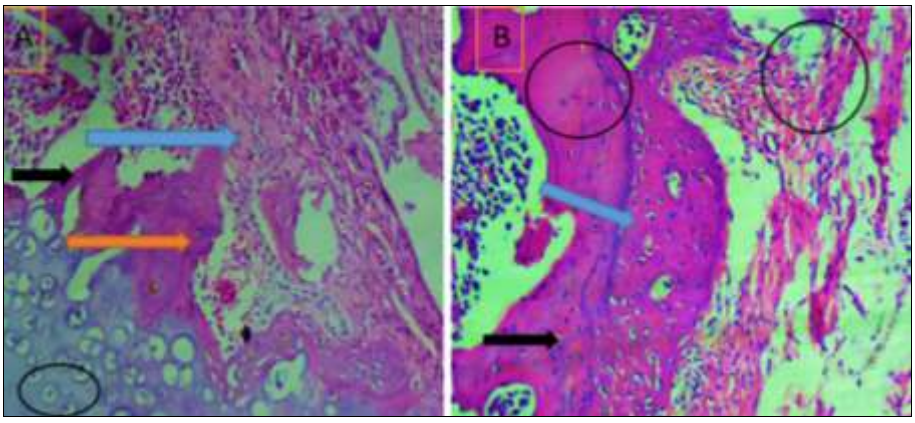

Fig 3: A: Caffeine treated group Zone of fracture typified by hypertrophic chondrocyte (black arrow) with scanty collagen and lamellar bone deposition (blue arrow) and blot clots, diffused angiogenesis few chondrocytes (black circle) and mononuclear cells. B: Control group Zone of fracture typified by presence of lamellar bone with numerous osteocytes, scanty collagen deposition, marked How ship lacunae and presence of few fibroblasts

Fracture Score

Table 1: The scoring system used to assess histological healing of fractures adopted from Sağliyan *et al.*, 2016 [12].

Score	Tissues Present
1	Empty Cavity
2	Fibrous tissues only
3	More fibrocartilage than Fibrous tissues
4	Fibrocartilage only
5	More Fibrocartilage than Bone
6	More Bone than Fibrocartilage
7	Bone Only

Table 2: Mean and standard deviation of Caffeine and control group

Days	Caffeine group	Control
14	1.2±0.4472[a]	1.2±0.4472 [a]
28	1.6±0.5477 [a]	2.6±0.4472 [a]
42	2.2±0.4472 [a]	4.4±0.5477 [a]

*Column means represented by the same superscript were not significantly different (p>0.05)

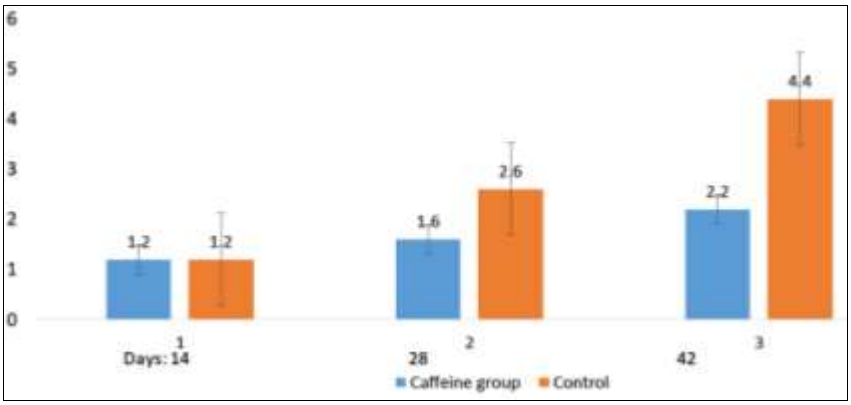

Fig 4: Bar chart Comparing Caffeine and Control group

Discussion

The fracture creation procedure in this study yielded minimal soft tissue reaction and absence of bone comminution. Closed reduction of fracture is now the most suitable model used to appraise fracture repair process, since the periosteum and the soft tissue surrounding the fracture site play a vital role during fracture repair process **(**Mana *et al*., 2022) [9]. Techniques such as osteotomy, bone drills and use of blunt guillotine-like apparatus to induce fracture are characterized by undesirable effects and recurrent complications including death, misplaced fracture, excess comminution and deep infection (Aurégan *et al.,* 2013; Haffner-Luntzer *et al.,* 2016) [13-14]. Comminutions arising from fractured bone varies in different degrees hence making it difficult to control during fracture healing (Pei and Fu, 2011; Ghiasi *et al.,* 2017) [15-16]. As the degree of fracture comminution can affect the formation of callus, there was association between the rat fracture model used with the resultant comminution and the soft callus produced. Inadequate stability of internal or external fixation leads to variable degrees of displacement and repeated movement of the bone fragments during the healing period (Glatt *et al.,* 2017) [18]. The guillotine-like apparatus technique used to create bone fractures in rat tibiae resulted in minor comminution but high displacement of fragments of bones, bending and pin angulation of more than 10° (Aurégan *et al.,* 2013) [13]. This technique produced consistent outcomes with minimal complications such as skin abrasion and laceration at the fracture site. Moreover, this technique allowed weight bearing by the fractured tibia soon after recovery from anaesthesia. This advantage was also recognized by Brumback *et al.,* (1999) [19] as the enabling advantage of closed fracture model.

In this study, we did not noticed any qualitative difference between the Caffeine treated group and the control group on day 14. This means that caffeine may not have any observable effect when administered simultaneously for 2 weeks post fracture. However on day 28, we noticed some qualitative changes with absence of collagen in the caffeine group even though it is not statistically significant, this agrees with Macedo *et al.,* (2015) [7] who states that prolonged intake of caffeine affects the bone by promoting an increase of calcium excretion, inhibition of osteoblast, fibroblast and chondroclast proliferation and delay in tissue repair process, which could also lead to an increased risk of fractures, osteoporosis, periodontal disease and may also affect successful fracture repair progression.

Furthermore, on day 42 the inflammatory cells, collagens and blood clots observed in the caffeine group suggest delay in fracture repair. This could be because treatments caffeine increases the levels of cAMP inside the inflammatory cells in the bone which may lead to decreased resorptive activity and possibly apoptosis of macrophages, which causes reduction in the secretory functions of T lymphocytes specifically by low production of gamma inteferon (γ-IFN) that stimulates the resorptive activities of macrophages as its main function (Heaney, 2002) [17]. These combined features may have resulted in the delay of the blood clot resorption and consequently impaired the fracture healing progression.

In conclusion, we found out that administration of caffeine for more than 2 weeks may affect collagen formation during fracture repair progression.

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