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Serum biochemical evaluation of healing of critical-sized long bone defects in rats treated with biphasic hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) bioceramic scaffolds#

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Abstract

Critical-sized long bone defects are those that would not heal spontaneously despite surgical stabilisation. The use of bioceramic scaffold has shown promising results in the repair of bone defects. The present study was undertaken to evaluate the serum biochemical parameters of Wistar rats treated for critical-sized segmental bone loss using biphasic hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) bioceramic scaffolds. The study was conducted in eighty male Wistar rats aged between 8-12 weeks, weighing 200-250 g body weight with critical-sized segmental defects in the femur. A 6 mm segmental mid-diaphyseal femoral defect was created under general anaesthesia. The bone defect was bridged with bioceramic scaffolds and retained in position with microplate and screws. Serum biochemical parameters serum calcium, phosphorous, acid phosphatase and alkaline phosphatase were evaluated four weeks before surgery, immediately after surgery and 4th, 8th, 12th, and 16th week after surgery. The evaluation of both serum calcium and phosphorous were found to be reliable indicators of new bone formation and mineralisation, whereas the evaluation of both serum acid phosphatase and alkaline phosphatase were found to be reliable indicators of bone healing during the treatment of critical-sized long bone defects in rats using biphasic hydroxyapatite and β-tricalcium phosphate bioceramic scaffolds.

Keywords: *Beta-tricalcium phosphate, bioceramic scaffold, hydroxyapatite, serum acid phosphatase, serum alkaline phosphatase, serum calcium, serum phosphorous.*

Critical-sized long bone defects are those that would not heal spontaneously despite surgical stabilisation (Reichert, 2010). Such fractures often fail to heal by normal bone healing. Hence, such bone injuries require scaffolds that promote the bridging of fracture fragments (De Coster *et al.,* 2004). Hydroxyapatite (HA) ceramics are widely used as bone substitutes due to their osteo-conductivity and bio-compatibility (Cacciotti, 2014). The disadvantage of plain hydroxyapatite was its slow rate of biological interaction (Thian *et al*., 2006). Biphasic calcium phosphate bioceramics composed of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) have ideal properties of synthetic bone graft such

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as tuneable resorption, bioactivity and intrinsic osteo-induction (Rompen *et al.,* 2006). The changes in bone mineral density after fracture alter biomarkers of bone activity which may be used as reliable indicators of new bone formation, mineralization and fracture healing Rueff-Barroso *et al.* (2008).

The present study was undertaken to evaluate the serum biochemical parameters of Wistar rats during treatment for critical-sized segmental bone loss using biphasic hydroxyapatite and β-tricalcium phosphate bioceramic scaffolds. Estimation of serum levels of calcium, phosphorous, acid phosphatase and alkaline phosphatase at varying time points are commonly used tests to evaluate biochemical changes during fracture healing.

Materials and methods

Eighty adult male Wistar rats with ages between 8-12 weeks, weighing 200-250 grams body weight were selected for the study. The experimental animals were procured from the Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences (CVAS), Mannuthy, and maintained as per the guidelines of CPCSEA and PCA Act 1960 (Amendment: 1998, 2001 and 2006) (Pandey and Sharma, 2011). Pre-surgical evaluation of the rats was conducted and all the rats were subjected to surgical creation of critical-sized defects in one femur under general anaesthesia. The femoral diaphysis was approached as per Costa *et al.* (2011). A 6 mm segmental mid-diaphyseal femoral defect was created under general anaesthesia. The bone defect was bridged with biphasic hydroxyapatite and β- tricalcium phosphate bioceramic scaffolds and retained in position with microplates and screws. The rats were maintained on uniform feeding and management conditions and observed for a period of 16 weeks. Analgesics and antibiotics were administered during the immediate post surgery period. The surgical wounds were dressed post surgery and skin sutures were removed after two weeks. The experimental animals were grouped into Group I, Group II, Group III and Group IV based on the time point of euthanasia (4^{th}, 8^{th}, 12^{th}, and 16^{th} week after surgery). The blood samples for the separation of serum were collected four weeks before surgery, immediately after surgery, 4^{th}, 8^{th},12^{th}, and 16^{th} week after surgery (T0, T1, T2, T3, T4 and T5). Serum biochemical parameters such as calcium, phosphorous, alkaline phosphatase, and acid phosphatase were determined using a semi-auto biochemical analyser. Serum calcium was estimated by a modified OCPC method using a calcium kit. Serum phosphorous was estimated by phosphomolybdate methodology using an inorganic phosphorus kit. Serum acid phosphatase was estimated by DGKC-SCE recommended procedure using an acid phosphatase kit. Serum alkaline phosphatase was estimated by DGKC-SCE recommended procedure using an alkaline phosphatase kit.

The acquired data was collected and analysed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Descriptive statistics are given by mean ± standard error of the mean. Group comparisons were performed by one-way ANOVA. P-values < 0.05 (significance level α = 0.05) were declared as statistically significant different (Prodinger, *et al.*, 2018).

Result and discussion

Serum calcium estimation

In Group I, the mean value of serum calcium (mg/dL) decreased from 9.27±0.08 at four weeks before surgery to 9.17±0.08 immediately after surgery and then increased to 13.91±0.08 by four weeks after surgery (Fig. 1, 3).

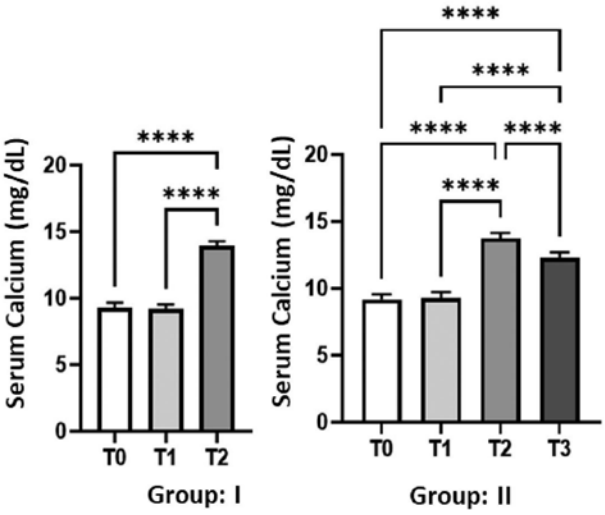

Fig. 1. Serum calcium estimation in Group I and Group II.

In Group II, the mean value of serum calcium (mg/dL) increased from 9.15±0.08 at four weeks before surgery to 9.28±0.09 immediately after surgery to 13.73±0.09 at four weeks after surgery. Thereafter, it decreased to 12.29±0.08 by eight weeks after surgery (Fig. 1, 3).

In Group III, the mean value of serum calcium (mg/dL) decreased from 9.26±0.10 at four weeks before surgery to 9.15±0.09 immediately after surgery and then increased to 13.86±0.09 at four weeks after surgery. Thereafter, the value decreased to 12.11±0.09 by eight weeks and 10.72±0.10 by twelve weeks after surgery (Fig. 2, 3).

In Group IV, the mean value of serum calcium (mg/dL) increased from 9.24±0.09 at four weeks before surgery to 9.25±0.09 immediately after surgery and to 13.74±0.11 by four weeks after surgery. Thereafter, the mean value decreased to 12.32±0.09 by eight weeks, 10.73±0.09 by twelve weeks and 9.09±0.11 by sixteen weeks after surgery (Fig. 2, 3).

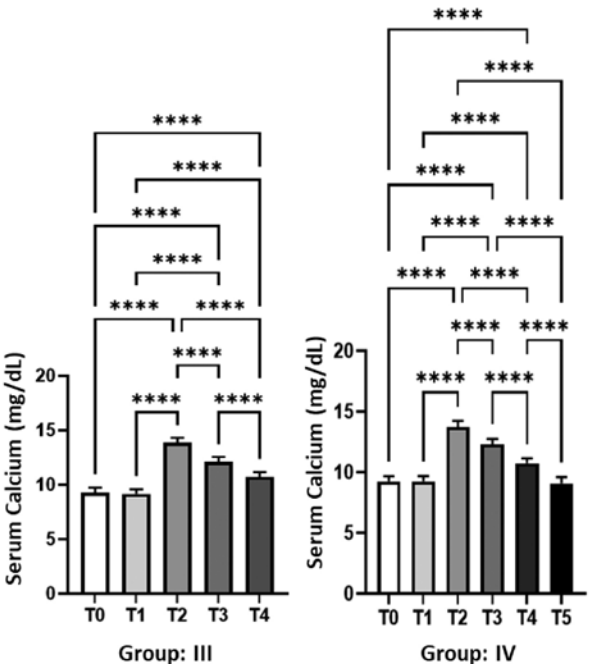

Fig. 2. Serum calcium estimation in Group III and Group IV.

The serum calcium level remained stable from four weeks before surgery to the day of fracture creation and peaked at four weeks after fracture creation. This may be attributed to the release of calcium into the circulation as a result of fracture. This finding was in line with the observations made by Kumar *et al.* (2013), who noticed an increase in serum levels of calcium in the second and third week after fracture as compared to the first week. This finding was also in alignment with the report by Rueff-Barroso *et al.* (2008), who reported that serum levels of calcium increased during stages of bone resorption. The serum level of calcium decreased steadily from the fourth week after fracture reaching the normal values by the sixteenth week after fracture treatment. This could be attributed to the deposition of calcium phosphate at the fracture site as a part of bone healing. This finding was consistent with the findings of Cavins (1924).

Serum phosphorus estimation

In Group I, the mean value of serum phosphorus (mg/dL) decreased from 7.21±0.07 at four weeks before surgery to 7.09±0.09 immediately after surgery and increased to 10.57±0.08 by four weeks after surgery (Fig. 4, 6).

In Group II, the mean value of serum phosphorus (mg/dL) decreased from 6.95±0.07 four weeks before surgery to 6.92±0.06 immediately after surgery. The mean value then increased to 10.51±0.08 by four weeks after surgery. Thereafter, it decreased to 9.48±0.07 by eight weeks after surgery (Fig. 4, 6).

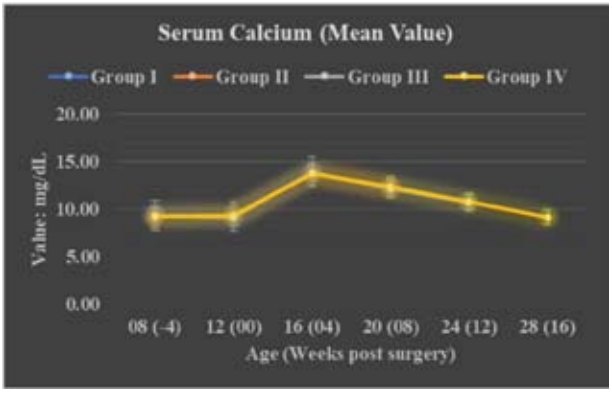

Fig. 3. Serum calcium estimation in Group I, II, III and IV

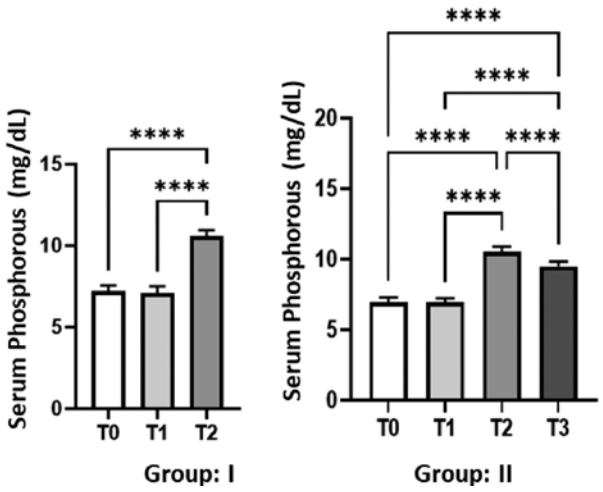

Fig. 4. Serum phosphorous estimation in Group I and Group II.

In Group III, the mean value of serum phosphorus (mg/dL) decreased from 7.13±0.07 four weeks before surgery to 7.02±0.08 immediately after surgery and then increased to 10.62±0.08 by four weeks after surgery.

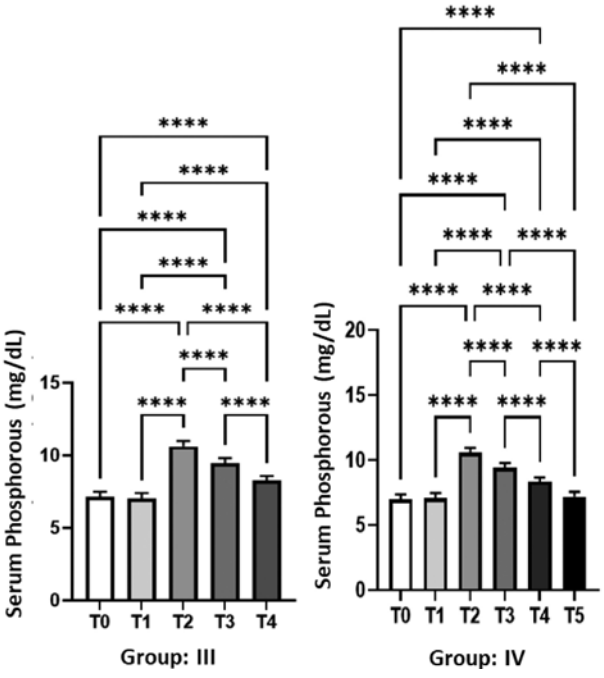

Fig. 5. Serum phosphorous estimation in Group II and Group IV.

Thereafter, it decreased to 9.45±0.08 by eight weeks to 8.28±0.06 by twelve weeks after surgery (Fig. 5, 6).

In Group IV, the mean value of serum phosphorus (mg/dL) increased from 6.99±0.08 four weeks before surgery to 7.09±0.08 immediately after surgery and to 10.56±0.08 by four weeks after surgery. Thereafter, it decreased to 9.41±0.08 by eight weeks, 8.36±0.06 by twelve weeks and 7.20±0.07 by sixteen weeks after surgery (Fig. 5, 6).

The serum phosphorus level remained stable from four weeks before surgery to the day of fracture creation and increased thereafter, peaking at four weeks after fracture creation. This may be attributed to the release of phosphate into the circulation as a result of bone fracture. The serum level of phosphorus decreased steadily from the fourth week after fracture stabilization and reached the normal value by the sixteenth week after fracture treatment. This may be attributed to the deposition of calcium phosphate at the fracture site as a result of callus mineralisation. These findings were in accordance with that of Cavins (1924) and Kumar *et al.* (2013), who noticed an increase in serum levels of phosphorus in the first and second week after fracture, which decreased during the third week. Rueff-Barroso *et al.* (2008), reported that serum levels of phosphorous increased during the stages of bone resorption, supporting the findings of current clinical trials.

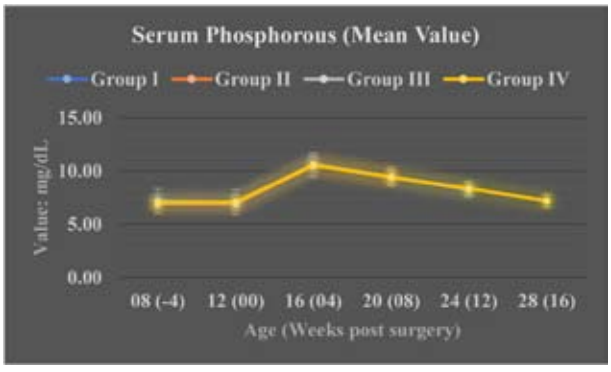

Fig. 6. Serum phosphorous estimation in Group I, II, III and IV.

Serum acid phosphatase

In Group I, the mean value of serum acid phosphatase (IU/L) increased from 2.98±0.29 at four weeks before surgery to 3.31±0.22 immediately after surgery. The mean value then decreased to 3.03±0.24 by four weeks after surgery (Fig. 7, 9).

In Group II, the mean value of serum acid phosphatase (IU/L) increased from 3.13±0.25 at four weeks before surgery to 3.43±0.19 immediately after surgery. The mean value then decreased to 3.33±0.22 by four weeks after surgery and increased thereafter to 4.81±0.21 by eight weeks after surgery (Fig. 7, 9).

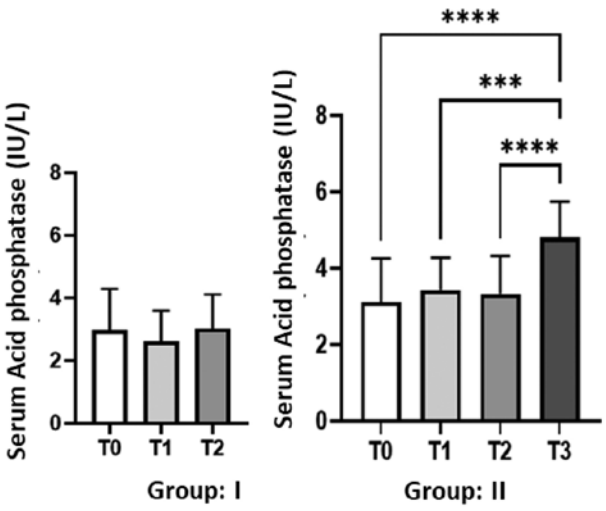

Fig. 7. Serum acid phosphatase estimation in Group I and Group II.

In Group III, the mean value of serum acid phosphatase (IU/L) increased from 2.88±0.22 at four weeks before surgery to 3.17±0.26 immediately after surgery. The mean value then decreased thereafter to 2.80±0.24 by four weeks after surgery. The mean value increased subsequently to 4.89±0.23 by eight weeks after surgery. Thereafter, it decreased to 4.46±0.15 by twelve weeks after surgery (Fig. 8, 9).

In Group IV, the mean value of serum acid phosphatase (IU/L) increased from 2.75±0.28 at four weeks before surgery to 3.07±0.29 immediately after surgery. The mean value decreased subsequently to

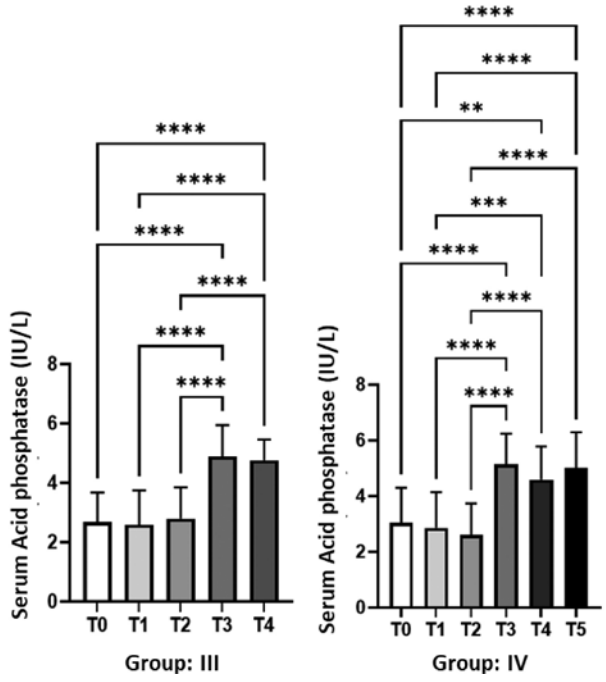

Fig. 8. Serum acid phosphatase estimation in Group III and Group IV.

2.61±0.25 by four weeks after surgery. The mean value then increased thereafter to 5.15±0.24 by eight weeks after surgery and then decreased to 4.59±0.27 by twelve weeks after surgery. A hike in the mean value was noticed thereafter to 5.01±0.29 by sixteen weeks after surgery (Fig. 8, 9).

In the present study, serum acid phosphatase levels showed a rise in the initial period. This might be due to the initial osteoclastic activity to remove necrotic bone tissue at the fracture margins (Stoffel *et al.,* 2007). The serum acid phosphatase level increased drastically from four weeks post surgery to eight weeks post surgery. This could be attributed to the osteoblastic activity and mineralisation at the fracture healing site. The serum levels then mildly decreased from the eighth week post surgery to the twelfth week post surgery. The serum levels then increased steadily from the twelfth week postoperative till the sixteenth week postoperative. This could be attributed to osteoclastic and osteoblastic activity and mineralisation as a part of bone remodelling at the site of fracture healing. These findings were in agreement with the findings of Veitch *et al.* (2006).

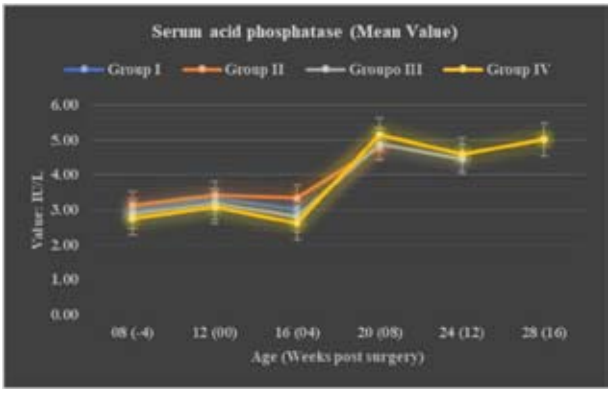

Fig. 9. Serum acid phosphatase estimation in Group I, II, III and IV.

Serum alkaline phosphatase estimation

In Group I, the mean value of serum alkaline phosphatase (IU/L) increased from 223.06±3.46 by four weeks before surgery to 296.49±8.17 immediately after surgery and to 801.93±4.11 by four weeks after surgery (Fig. 10, 12).

In Group II, the mean value of serum alkaline phosphatase (IU/L) increased from 218.76±2.06 by four weeks before surgery to 308.48±6.60 immediately after surgery and to 801.80±6.01 four weeks after surgery. Thereafter, it was found reduced to 703.15±7.00 by eight weeks after surgery (Fig. 10, 12).

In Group III, the mean value of serum alkaline phosphatase (IU/L) was found to increase from 231.01±3.08 by four weeks before surgery to 295.68±7.14 immediately after surgery and to 794.65±5.68 by four weeks after surgery. Thereafter, it was found to decrease

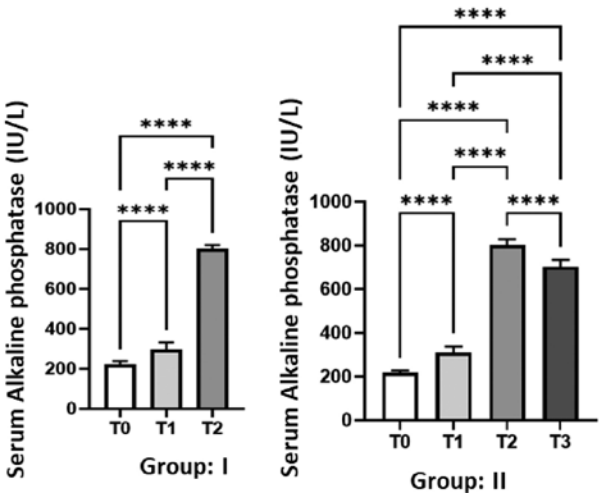

Fig. 10. Serum alkaline phosphatase estimation in Group I and Group II.

to 713.18±6.16 by eight weeks and to 607.96±7.35 by twelve weeks after surgery (Fig. 11, 12).

In Group IV, the mean value of serum alkaline phosphatase (IU/L) was found to increase from 222.22±3.45 by four weeks before surgery to 305.06±5.58 immediately after surgery and to 805.36±6.48 by four weeks after surgery. Thereafter, it was found to decrease to 695.76±6.00 by eight weeks, 589.15±5.42 by twelve weeks and 495.75±7.58 by the sixteenth week after surgery. The serum alkaline phosphatase activity showed higher values than pre-fracture values during the entire period of observations, similar to the observations made by Dinesh (2018) (Fig. 11, 12).

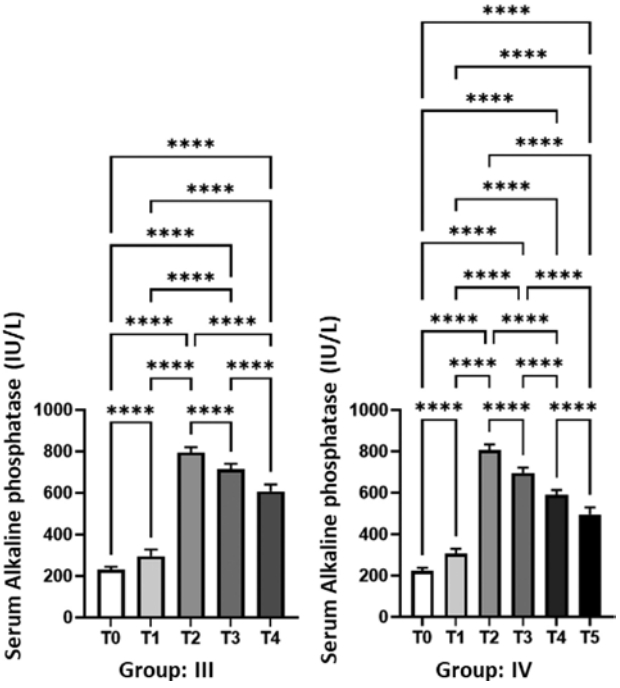

Fig. 11. Serum alkaline phosphatase estimation in Group III and Group IV.

The serum alkaline phosphatase level which was high at four weeks before surgery (as a result of normal bone growth), increased drastically from the day of fracture creation and peaked at four weeks after stabilisation of fracture. The levels then decreased steadily from the fourth week post surgery till the sixteenth week post surgery. These observations were contrary to those made by Nakagawa *et al.* (2006), who observed a maximum concentration of ALP three weeks after fracture which returned normal by the tenth week.

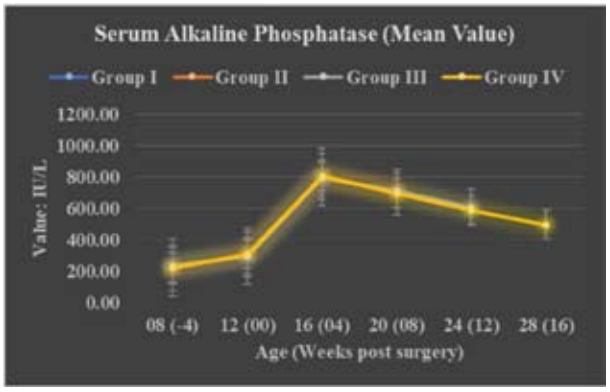

Fig. 12. Serum alkaline phosphatase estimation in Group I, II, III and IV.

Golub and Boesze-Battaglia (2007) reported that ALP is an enzyme that is expressed by osteoblasts during bone formation which increased during the early stages of bone formation and decreased as bone remodelling occurred. It increased the local concentration of inorganic phosphate (mineralisation promoter) and decreased the concentration of extracellular pyrophosphate (mineralisation inhibitor).

Atalay *et al.* (2015), Wang *et al.* (2012) and Kisiel *et al.* (2012) reported that the alkaline phosphatase level acts as a marker for the course and rate of bone healing. Its activity indicates the differentiation of cells with osteogenic potential into mature osteoblasts. It is also noted that calcification does not occur without a change in ALP activity.

The findings of the current research work also fit into the scheme of biochemical processes reported by Sousa *et al.* (2011), who observed increased serum alkaline phosphatase activity from the baseline values till the twelfth week after fracture, which reduced to normal reference levels thereafter. The persistent higher levels of ALP may be due to the continuing osteoblastic activity during the bone remodelling process.

Conclusion

Biphasic hydroxyapatite and β-tricalcium phosphate bioceramic scaffolds have demonstrated promising results in promoting bone regeneration in critical sized long bone defects in rats. The study revealed that a change in bone mineral density after fracture alters the levels of serum calcium, phosphorous, serum acid and alkaline phosphatase enzymes. The study revealed that the levels of serum calcium were indirectly indicative of the extent and quality of new bone formation, whereas the levels of serum phosphorous were indirectly indicative of bone mineralisation. The evaluation of both serum acid phosphatase levels and serum alkaline phosphatase levels were found to be reliable indicators of bone healing during the treatment of critical-sized long bone defects in rats using biphasic hydroxyapatite and β-tricalcium phosphate bioceramic scaffolds.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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