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RESEACH ARTICLE

THE MODULATORY ROLE OF HYDROXYPROLINE, ALKALINE PHOSPHATASE, CALCIUM AND CREATININE IN FRACTURE HEALING AMONG MALE PATIENTS IN ESAN LAND, EDO STATE, NIGERIA

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Abstract

Background: Fracture healing is an extremely complex interaction of cells, biological pathways, and molecules. Interference with any of these pathways or proteins either promotes or, more likely, decreases fracture healing. The inflammatory response is certainly one of the initiating factors for bone healing; this phase is a critical period characterized by low oxygen tension, impaired perfusion, and migration of a wide array of cells and the release of active molecules. When searching for biochemical bone markers, molecules in bone of connective tissue origin that may be detected in plasma and/or urine are being targeted; as they provide a dynamic view of the remodeling process of bone. Bone turnover markers (BTMs) are a product of bone cell activity. They are a series of protein or protein derivative biomarkers released during bone remodeling by osteoblasts or osteoclasts. Bone resorption markers are related to osteoclast resorption of the matrix and include tartrate-resistant acid phosphatase and degradation products of type I collagen, especially urinary hydroxyproline (OHPr), etc. **Aim of the Study:** The aim of the study is to investigate early detection of fracture healing disturbances among patients with fractures. **Materials and Methods:** A total number of fifty (50) male subjects aged between 20 and 45 were used in the study. They were divided into two groups of 25 people each (control and test groups). OHPr was determined using an automated method based on Stegmann-Staeder's method as modified by Utevskaya and Persky, and serum calcium concentrations by O-cresol phthalein complexone method. Serum creatinine (Cr) was estimated by modified Jaffe's method; and alkaline phosphatase by enzymatic method. **Results:** It was found that biosynthesis of collagen in the 6th week period of healing increased due to an increase in (OHPr) content, which subsequently decreased to normal reference interval at the 9th week. Serum alkaline phosphatase activity in healing fractures increased significantly during the first two weeks of the healing process and subsequently decreased to normal reference interval at the 9th week. At the same time, serum calcium increased up to the 3rd week after fracture and then returned to normal level within the next weeks of fracture. There was no significant change in creatinine levels across the weeks. **Conclusion:** The increase in the biosynthesis of collagen protein during the healing period was due to the increase in the content of its index marker, hydroxyproline. Serum alkaline phosphatase activity in healing fractures increased significantly and subsequently decreased to normal reference interval. At the same time, serum calcium increased after fracture and then returned to normal level within the next weeks of fracture. There was no significant change in creatinine, which could be due to reduced GFR.

Keywords: Alkaline phosphatase, collagen, fracture, hydroxyproline, serum calcium, serum creatinine

Introduction

Fractures occur when the physical force exerted on bone is stronger than the bone itself. Bone healing is defined as the functional stage of bone regeneration after a trauma, enabling the bone to acquire its physio-anatomical (load-carrying properties) without additional assistance.[1] The expected time required for a complete and successful fracture healing process depends on factors such as degree of injury of adjacent soft tissues; displacement of fracture ends and the degree of comminution; the selected method for the stabilization of the fracture; the age of the patient; and the presence of co-morbidities (diabetes, tobacco use, malnutrition and prolonged use of non-steroidal anti-inflammatory drugs). It is estimated that 5-10% of all patients with long bone fractures develop impaired fracture healing processes, especially delayed union and non-union processes (Amir., *et al* 2021).

Fracture healing is an extremely complex interaction of cells, biological pathways, and molecules. Interference with any of these pathways or proteins either promotes or, more likely, decreases fracture healing. The inflammatory response is certainly one of the initiating factors for bone healing. This phase is a critical period characterized by low oxygen tension, impaired perfusion, and migration of a wide array of cells and the release of active molecules (Einhorn. and Gerstenfeld, 2015).

When searching for Biochemical bone markers (BTMs), molecules in bone of connective tissue origin that may be detected in plasma and/or urine are being targeted, as they provide a dynamic view of the remodeling process of bone (Einhorn. and Gerstenfeld, 2015). The BTMs are products of bone cell (osteoclast) activity. They are a series of protein or protein derivative biomarkers released during bone remodeling by osteoblasts or osteoclasts and are generally subdivided into three categories: bone resorption markers, bone formation markers, and osteoclast regulatory proteins. They are further categorized into matrix products that are liberated during bone resorption or formation or cellular products that are directly secreted into the circulation at levels commensurate with the number or activity of osteoclasts or osteoblasts (Fig. 1) (Xu. and Teitelbaum, 2013). Haematological and serological BTMs have been studied in fracture healing research to monitor fracture callus development. They have provided prognostic value for early detection of fracture healing complications (Ruisen., *et al* 2021 Tralman., *et al* 2013; Hans-Chrristophe, Ralph. and Catherine, 2010).

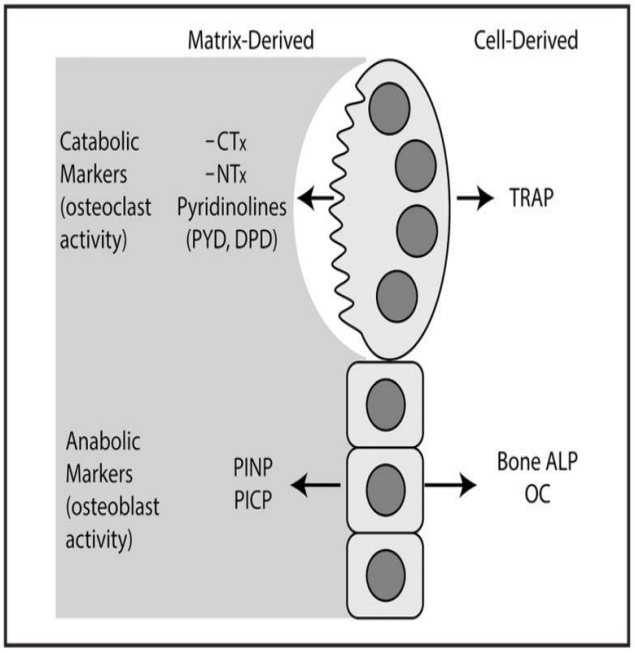

Figure 1: Categories of BTMs (Greenblatt., *et al* 2017)

Hydroxyproline (4-OHPr) is a non-proteinogenic amino acid formed by the post-translational hydroxylation of proline, and is a major component of collagen, in which it serves to stabilize the helical structure. It accounts for 13-14% of collagen's total amino acid content (Ivan., *et al* 2018; Gladstone., *et al* 2014). The hydroxyproline of animal organisms is thought to be confined almost exclusively to the connective tissue scleraproteins, collagen, and elastin (Masahiro., *et al* 2020; Jenna, Nancy, Kadie. and Chartrisa, 2017). Kidney-processed urinary 4-OHPr is derived partly from collagen or gelatin-containing foods in the diet and partly from the breakdown of collagen in the body. Excretion of endogenous 4-OHPr reflects the rate of collagen degradation. Iits level can be used as an indicator of both the presence and overall metabolism of collagen (Bishnu. and Eric, 2014).

Conditions that increase collagen turnover can elevate urine OHPr levels. It has been shown that unlike most other proteins, the turnover of collagen is extremely slow and can, therefore, reflect age-related changes

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(Bielajew, Hu. and Athanasiou, 2020; Huije., *et al* 2013). The total reference range of hydroxyproline in the urine among humans aged 18-21 years is 13-28 mg/24/m^2; 22-55 years of age, 8.5-23.5 mg/24/m^2 (Bishnu. and Eric, 2014).

Creatinine (Cr) ($C_4H_7N_3O$) is a non-protein amino acid that is synthesized mainly in the liver, kidneys, and pancreas (Brosnan. and Brosnan, 2007). However, other tissues (e.g., brain and testes) are also able to produce Cr (Béard. and Braissant, 2010; Brosnan, da Silva. and Brosnan, 2011). Most of the Cr pool is found in tissues with high-energy demands such as the skeletal muscles. Creatinine is also a breakdown product of the metabolism of dietary meat (Nair., *et al* 2014; Brosnan. and Brosnan, 2007). The type of Cr in a skeletal muscle (creatinine phosphate), depending on muscle mass, is usually produced at a fairly constant rate by the body. Creatinine is critically important in assessing renal function because it has several interesting properties. Except for extreme muscle exertion or muscle breakdown, Cr has a stable plasma concentration and is released into circulation at a relatively constant rate. Since Cr is chemically the anhydride (dehydration product) of Cr, once formed, it cannot be converted back into Cr. Therefore, the amount of Cr formed daily is related to the muscle mass, which varies with ethnicity, age, and gender (Diego., *et al* 2021). Its clearance levels go lower as a person ages and are also based on a person's size. Normal Cr clearance levels for men under the age of 40 are between 107 and 139 millilitres per minute (500-2000 mg/day) (Diego., *et al* 2021). For men, normal urine Cr levels fall between 0.8 and 1.8 grams per day; and for women, the values fall between 0.6 and 1.6 grams per day. Generally, levels go down 6.5 millilitres per minute for every 10 years after a person turns 20 (Diego., *et al* 2021; Onwuka., *et al* 2021). Since Cr is produced and removed at a relatively constant rate, the amount of urinary Cr can be compared to the amount of another substance being measured. This stable excretion rate is useful when evaluating both 24-hour urine samples and random urine samples (AACC, 2001-2017). Young muscular or middle-aged adults may have more Cr in their blood than what is the norm for the general population; elderly persons, on the other hand, may have less Cr in their blood than what is the norm.

Alkaline phosphatases ($Zn_AZn_BMg_C)_2$ (ALPs) are a group of isoenzymes located on the outer layer of the cell membrane. They catalyze the hydrolysis of organic phosphate esters found in the extracellular space. Zinc and magnesium are essential cofactors of this enzyme.

ALP is found in the cytosol of liver cells and the canalicular membrane of hepatocytes (Green. and Sambrook, 2020). ALP is found in decreasing concentrations in various organs such as the placenta, ileal mucosa, kidney, bone, and liver. Over 80% of the ALP in serum originates from the liver and bone, with minor contributions from the intestine. Although ALPs are present in various tissues throughout the body, their precise physiological function remains elusive (Vimalraj, 2020).

The ALPs are categorized into 2 types—tissue-specific and tissue-nonspecific. Tissue-specific ALPs are exclusively present in the intestine, placenta, and germinal tissue (Azpiazu, Gonzalo. and Villa-Bellosta, 2019), specifically within the tissues where they are expressed under physiological conditions. Under specific conditions, the tissue-specific ALPs may also contribute to the circulating pool of serum ALP when there is increased stimulation of their production. The tissue-nonspecific ALPs are clinically significant, as they constitute most of the circulating fraction in serum (Zaher., *et al* 2020).

The Study Area – Esan land has an area of 2,814,347 square kilometres. It lies between the fringes of the Savannah to the North and the rain forest to the South. Geographically, Esan land is located at Longitude 605 degree Celsius and Latitude 605 degrees Celsius. It has boundaries to the south with Benin, to the South-east with Agbo, to the North and East with Etsako, and to the West with the River Niger.

Figure 3: Map of Esan Land

There is paucity in the number of parameters investigated in many studies in the healing process of bone fracture. It is, therefore, important to carry out this research that employs the parameters herein. The aim of this study is, therefore, to investigate the early fracture healing process among male patients in Esan Land, Edo State, Nigeria.

AIM OF THE STUDY

The aim of the study is to investigate early detection of fracture healing disturbances among patients with fractures.

OBJECTIVES OF THE STUDY

To achieve the aim, the following tasks were set: measure the concentration of 24-hour urinary OHPr; estimate the content of alkaline phosphatase; measure the concentration of serum calcium; and determine the concentration of creatinine

Ethical clearance on all components of the research (№ 004/23 (001/23) was obtained from the 'Ethical Committee' of the College of Medicine, Ambrose Alli University, Ekpoma, Nigeria.

MATERIALS AND METHODS

Design of the Study

A total number of fifty (50) male subjects aged between 20 and 45 were divided into two (2) groups:

Group 1: A control group that consists of twenty-five (25) healthy males.

Group 2: A test group made up of twenty-five (25) male patients with minimally displaced or undisplaced long bone fractures.

Briefly, after obtaining an informed consent, all patients were followed up for 9 weeks. Fasting blood (to estimate serum creatinine, calcium and alkaline phosphatase concentrations), as well as 24-hour urine samples (to estimate the content of hydroxyproline), were collected.

STATISTICAL ANALYSIS

The data were analyzed using software, version 20.0 (SPSS) and the results were expressed as mean values and standard deviations. Analysis of variance (ANOVA) was performed to test the differences between groups. Differences were considered significant where $p<0.05$.

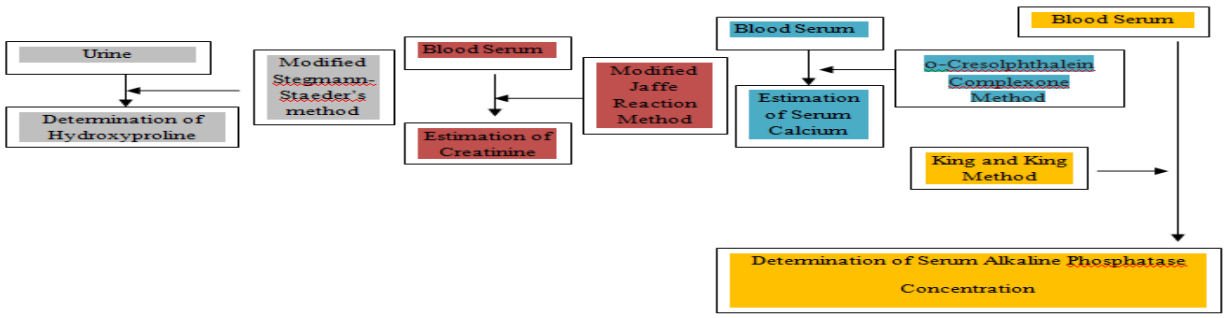

Figure 2: Flowchart of the Research Methodology

Analytical Determination of Hydroxyproline

Modified Stegmann-Staeder's method was used to measure the concentration of OHPr; the method is based on the condensation reaction of products obtained from the interaction of OHPr oxidation (pyrole) with para-dimethylaminebenzoaldehyde (DABA) (Utevskaya. and Persky, 1982). Ultimately, the coloured products with maximum light absorption of 540nm were measured using a spectrophotometer. The reference interval of urinary total hydroxyproline is 19–36 mg/day.

Estimation of Serum Creatinine – *A* concentration of creatinine was measured by modified Jaffe reaction method (Moore. and Sharer, 2017; Jose. and Salazar, 2014). The principle of the method is that creatinine in samples reacts with picric acid in an alkaline medium forming a red-orange coloured creatinine-picric acid, complex, which absorbs at 510nm. The rate of the formation of colour is proportional to the creatinine concentration in the sample.

Estimation of Serum Calcium

The o-cresolphthalein complexone (oCPC) method was used in measuring the serum calcium level. The method is based on the reaction between calcium and o-cresolphthalein complexone in the presence of 8-hydroxyquinoline-5-sulfonic acid to form a deep purple complex (Mohan, 2020; Bo. and Westlin, 1972).

Determination of Serum Alkaline Phosphatase Concentration

Principle of the Method: The kinetic method uses p-nitrophenyle phosphate (PNPP) as a substrate. The PNPP does not absorb at the wavelength chosen to read the test (405nm). Alkaline phosphatase cleaves phosphate; free p-nitrophenol (PNP) is formed and converted to a yellow colour at an alkaline pH. The optimum pH for alkaline phosphatase is about 10. The rate of formation of the yellow colour was used to measure enzyme activity.

Procedure: The level of alkaline phosphatase (ALP) was determined using the method of King and King (1984). The ALP catalyzes the hydrolysis of phenyl phosphate, and the hydrolysis product of phenol is condensed with 4-aminoantipyrene and then oxidized with alkaline to give a complex that was measured at 500nm.

RESULTS AND DISCUSSION

The results of the study are represented in tables 1-3. In the process of normal fracture healing, alkaline phosphatase might be associated with osteoblast activity during the early stage of healing (Table 1), whereas osteocalcin may be with the mineralization of the woven bone during the late stage (Osipov., *et al* 2018). Alkaline phosphatase (ALP) activity may serve as a marker of the rate of bone healing after sustained fractures. Observed increased production of collagen was also found soon after fracture (Table 1). This is associated with an increase in the concentration of hydroxyproline. Bone resorption markers increased soon after fracture, and bone formation markers gradually increased thereafter (Osipov., *et al* 2018).

Serum ALP and urinary OHPr significantly increased after treatment up to the 6^{th} week and then decreased to a normal reference interval at the 9^{th} week (Table 2). The former reached **a** lower level of reference interval in this group. It was observed that serum ALP activity significantly increased during the period in the fractured patients compared to the control. This agrees with the results of a study conducted by Singh *et al.*, (2013) who also observed significantly higher levels of serum ALP activity in the group of patients that have fractures compared to the control group. The results also agree with reports found by Uziel *et al.*, (2009) who demonstrated how alkaline phosphatase activity in healing fractures in rats increased significantly during the first two weeks of the healing process (Francesca., *et al* 2022). Bone ALP (BALP) and its liver isoforms represent the most relevant fraction of total ALP activity, with an almost equal contribution to about 95% of this enzyme. In the absence of pregnancy and liver isoforms or intestinal disorders, ALP activity could be an inexpensive marker for monitoring the bone fracture healing process.

While urinary OHPr decreased in week 9, its concentration in weeks 3 and 6 was significantly higher than in the control subjects ($p<0.05$) (Table 2). More so, significant ($p<0.05$) changes were observed across the various weeks. These findings were similar to those found by Mukhopadhyay *et al.*, (2011), which showed

that patients with fractures presented higher values of urinary OHPr at 3rd week after the fracture than those patients with impaired healing fractures (Mukhopadhyay, 2011). This might be because the excretion of OHPr in urine is regarded as a marker of bone resorption, and approximately 50% of the amino acid is derived from the degradation of bone collagen (Paietta, Burger. and Ferguson, 2013). An increase in the OHPr levels found soon after fracture in this study was suggestive of increased production of collagen (Paietta, Burger. and Ferguson, 2013). These modifications represent the changes in levels of total OHPr excretion in urine and ALP in plasma (Ivan., *et al* 2018). At the same time, it was recently shown that significant ($p<0.05$) differences in OHPr levels were observed between the control and the test groups with long bone fractures (Das., *et al* 2015).

Serum calcium significantly increased in the 3rd week after fracture and subsequently returned to the normal levels. The decrease was at a very slow rate across the weeks of study ($p>0.05$). This agrees with the findings of Petersen (1926), who reported that plasma calcium levels might decrease after fracture and that this prevents the healing process (Marx., *et al* 2023). At the same time, reports by Howard *et al*., (1945) showed that plasma calcium frequently increased somewhat after the fracture (Soumi, 2015). Contrarily, Speed (1931) found no changes in plasma calcium in fracture patients (Howard, Parson. and Bigham, Jr., 2014).

An insignificant ($p>0.05$) change in Cr levels was recorded (Table). This agrees with the findings of Garnero, *et al*. (1994), who observed that elevated serum Cr was commonly associated with reduced Glomerular Filtration Rate (GFR) (Fischer, Haffner-Luntzer, Amling. and Ignatius, 2018); normal biological variability of serum Cr; concurrent use of medications that inhibit tubular secretion or cause hemodynamic changes to renal perfusion; nutritional or supplemental intake of Cr or its increased serum generation as occurs in hereditary or acquired muscle disease. Artifactual increases could also occur because of interfering substances or the choice of measurement assay. It is important to note that the elevation of serum Cr is not a normal feature of aging (Rakesh, 2013).

Table 1: Concentrations of Serum Alkaline Phosphatase, Urinary Hydroxyproline, Serum Calcium and Creatinine in Control and Test Groups.

Parameter	Control	Fractured Subjects
Serum Alkaline Phosphatase (mg/dl)	79.22±15.23	105±44*
Urinary Hydroxyproline (mg/dl)	22.90±5.24	33.40±2.62*
Serum Calcium (mmol/l)	2.16±0.18	1.06±0.85*
Serum Creatinine (g/dl)	1.01±0.1	2.17±0.02

* = Significant Compared to the Control Group

Table 2. Weekly Concentrations of Serum Alkaline Phosphatase, Urininary Hydroxyproline, Serum Calcium and Creatinine in the Control and the Test Groups.

Parameters	Control	Week 3	Week 6	Week 9
Serum Alkaline Phosphatase. (IU/L)	79.22±15..23	104±7.20*	103±8.40*	87±7.70*
Urinary Hydroxyproline (mg/dl)	22.90±5.12	30.40±6.24*	27.20±6.06*	16.50±5.86
Serum Calcium (mmol/l)	2.16±0.183	3.09±0.79*	1.10±0.81	1.62±0.96
Serum Creatinine (g/dl)	1.01±0.15	1.23±0.03	1.72±0.02	2.4±0.01

* = Significant Compared to the Control Group

Table 3: Comparison between Different Weeks after Fracture Using Post Hoc Test (Boniferoni Test).

Weeks	Serum Alkaline Phosphatase	Urinary Hydroxyproline	Serum Calcium	Serum Creatinine
Week 3 vs Week 6	0.00*	0.00*	0.07	0.52
Week 3 vs Week 9	0.00*	0.00*	0.00*	0.00*
Week 6 vs Week 9	0.00*	0.00*	0.00*	0.40

* = Significant.

RECOMMENDATIONS

The study recommended the following:

1. Feeding patients with food rich in collagen should be encouraged.
2. More research work using a larger sample size should be used to validate the results obtained in this study.
3. Calcium supplement**s** should be given to patients.
4. Confirmatory test**s** for increased creatinine levels in patients should be carried out.
5. More work on the use of hydroxyproline should be carried out, as it has been discovered to play a major role in tissue remodeling.

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Conflict of Interest: None declared

Funding: None

Authors' Contributions:

OWO; carried out all experiments in Federal Eye Hospital Irua, Ondo State, Nigeria. FKI; provide technical support in the experimental protocol and data collection, EAB supported with the methods of hydroxyproline estimation, as well as made a lot of modifications in the manuscript; he is at the same time, the correspondence author to the submission of the manuscript.

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