

Original Research Article

Biochemical Indices of Bone Status in Patients with Epilepsy

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ABSTRACT

There is a growing awareness of effect of antiepileptic drugs on bone health in people with epilepsy. Long-term antiepileptic therapy can lead to reduction in bone mineral density. Biochemical abnormalities of bone metabolism including hypocalcaemia, hypophosphatemia, vitamin D insufficiency and increased alkaline phosphatase have been reported in epileptic patients. Consequently, identification of epileptic patients who have decreased bone strength and can be predisposed to bone fractures is an important issue in management of epilepsy. The main objective of the study was to assess bone mineral status of individuals of different age groups and sex of epileptics and non-epileptics. Serum calcium, phosphorus, creatinine, protein and albumin levels of epileptics and non-epileptics were observed to be in normal range. A significant increase in serum tartrate resistant acid phosphatase and alkaline phosphatase levels in epileptic males and females which is an indicator of biochemical abnormality of bone mineral homeostasis.

Key words: Biochemical indices, Bone status, Epilepsy.

INTRODUCTION

Epilepsy is one of the common neurological disorders affecting 50 million people worldwide (Shaker et al., 2009). Epidemiological studies have shown that patients with epilepsy are at higher risk of fractures when compared to the general population (Souverain et al., 2005). Bone is a metabolic active specialized cartilage tissue which undergoes the continuous remodeling by two counteracting processes viz., bone formation and destruction. Osteoblasts carry out the function of bone formation by secreting fiber and inorganic salts and thereby get transformed to osteocytes. These cells are responsible for the maintenance of bones. Osteoclasts are multinucleated and large cells responsible for the destruction/resorption of bone cells

(Starr and McMillan, 2001; Chatterji, 2003). Under normal conditions, balance in bone turnover is achieved with the help of hormones such as parathyroid, calcitonin, other steroid hormones, vitamin D and local mediators viz., cytokines, growth factors etc. On the other hand, somatic growth, ageing, metabolic bone diseases, states of increased and decreased mobility, therapeutic intervention, etc. are few conditions which create an imbalance in bone turnover (Mahan and Krause's, 2000; Groff and Gropper, 2000; Eastwood, 2003; Sander and Emery, 2003). It has been reported that 15 percent of fractures are pathological due to metabolic bone diseases (Jaglal et al., 1995; Vestergaard et al., 1999; Souverein et al., 2005). Other risk factors for osteopathy in epilepsy include

inadequate dietary intake of calcium, vitamin D insufficiency, immobilization, reduced exposure to sunlight, ageing, and sex (Gough et al., 1986; Riggs and Melton, 1986; Michael et al., 2003; ICMR, 2010).

There is a growing awareness of effect of Anti-epileptic drugs (AEDs) on bone health in people with epilepsy. These drugs alter Vitamin D metabolism through induction of cytochrome P₄₅₀ enzymes. In addition, they also interfere with intestinal absorption of calcium. Impaired absorption would lead to hypocalcaemia and hyper secretion of parathormone (PTH). Studies have reported that long term AED therapy results in reduced bone mineral density as these drugs have deleterious effects over bone health as they directly affect the bone cells responsible for the bone formation (Farhat et al., 2002; Babayigit et al., 2006; Valsamis et al., 2006; El-Hajj et al., 2008) which results in reduced bone mineral density and abnormalities in calcium metabolism including hypocalcemia, hypophosphatemia, elevated levels of serum alkaline phosphatase and parathyroid hormone; reduced levels of vitamin D metabolites and results in rickets and osteomalacia in children and adults, respectively (Feldkamp et al., 2000; Sato et al., 2001; Andresset et al., 2002; Pack, 2003; Boluk et al., 2004; Berqvist et al., 2008).

Studies have reported that life style in epilepsy patients is also responsible for poor bone health. Individuals suffering from epilepsy lead a sedentary and indoor life style which provokes seizures or risk during seizures. They are frequently associated with mild vitamin D deficiency (Henderson, 1997; Nakken and Tauboll, 2010; Santosh et al., 2014). Childhood and puberty is the critical periods of bone mineralization. Identification of epileptic patients who have decreased bone strength can be predisposed to bone fracture which is an important issue in management of epilepsy. The main objective of the study was to assess bone mineral status of epileptic and non-epileptic individuals of different age groups and sex.

MATERIALS AND METHODS

Selection of subjects - Inclusion and exclusion criteria

Epileptic subjects (n= 459) with an age range of 3 to \leq 80 years of both sexes (Males -270 and Females -189), below poverty line category, physically active patients on anti-epileptic drug treatment, regularly attending epilepsy clinic of the Super Specialty Hospital Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, were included in the study. Patients with comorbid illness such as gastrointestinal illness and chronic liver and kidney diseases were excluded in the study. Remaining subjects were (n=243; males - 106 and females -137) non-epileptics also belonged to below poverty line category and who were attending other outpatient clinics other than epilepsy were included in the study to compare their general health conditions with epilepsy patients with reference to certain parameters used for the study. None of the patients were on any supplementation tablets of calcium or vitamin D. The medical information relating to epileptic and non-epileptic patients was collected from Medical records of SVIMS, Tirupati. The collected information was recorded and kept safe for future reference.

Biochemical analysis

Collection of blood samples

Blood samples were collected from selected subjects after informed consent and their willingness to be a part of the study. Blood was collected from the subjects under the supervision of a trained doctor and technician from SVIMS, Tirupati. The collected blood samples were stored in cold conditions at the site of collection, processed and stored at 4° C till analysis of serum calcium, phosphorus, alkaline phosphatase, tartrate resistant acid phosphatase, creatinine and albumin using standard protocols.

Estimations of serum calcium

Serum calcium was estimated by the method of Clark and Collip (1925). To serum (2 ml) add 2 ml of distilled water and 1 ml of 4 per cent ammonium oxalate

solution and mix thoroughly by holding the tube at the mouth and giving it a circular motion by tapping the lower end. The tubes are allowed to stand for 30 minutes. Mix the contents again and centrifuge for 10 min at 1500 rpm. Decant the supernatant carefully and wash the precipitate with dilute ammonia solution. Place the tubes in hot water bath for 1 to 2 minutes and then titrate against 0.01 N KMnO₄ till definite pink color persists for at least minute and express Serum calcium as mg/dl.

Estimation of serum inorganic phosphorus

Serum phosphorus was estimated by the method of Fiske and Subbarao (1925). To serum (1 ml) add 4 ml of 10 % trichloroacetic acid mix well. Add 1 ml of 2.5 % ammonium molybdate solution and mix, 0.4 ml of the aminonaphtho sulphonic acid and make up the volume to 10 ml with distilled water. 5 ml of the standard phosphate solution was taken into a suitable test tube and mixed. Allow the tubes to stand for 5 minutes and read the absorbance using a red filter at 680 nm. Treat standard phosphorus solution having different concentration (50µg/ml) in the same way and express serum phosphorus as mg/dl.

Estimation of serum alkaline phosphatase

Serum alkaline phosphatase was estimated by the method of King and Armstrong (1934). To serum (0.1 ml) add 2 ml of buffer substrate was and place in a water bath at 37° C for a 15 minutes. Remove the tubes from the water bath; add 0.8 ml of 0.5 N sodium hydroxides and 1.2 ml of 0.5 M sodium bicarbonate. Add 1 ml of 0.6 % amino-antipyrine solution and 1.0 ml of 2.4 % potassium ferricyanide solution. Treat standard phenol solution (0.01 mg/ml) with different concentrations in the same way. Read the absorbance at 520 nm and express serum alkaline phosphatase as IU/L.

Estimation of serum creatinine

Serum creatinine was estimated by the method of Brod and Sirota (1948). To serum (2 ml) add 2 ml of distilled water and precipitated the proteins by adding 2 ml of 5% sodium tungstate and 2 ml of 2/3 N sulphuric acid, and allow it to stand for 10

min and filter. To 3 ml of the filtrate, add 1 ml of 0.92 % of picric acid solution and 1 ml of 0.75 N sodium hydroxide solutions. Allow the mixture to incubate for 15 min and read the developed color at 540 nm. Treat standard creatinine solution (0.5 mg/ml) with different concentrations in the same way and express serum creatinine as mg/dL.

Estimation of serum tartrate resistant acid phosphatase (TRACP)

Serum tartrate resistant acid phosphatase was estimated by the method of Otto *et al.*, (1946). Take two set of tubes. Add 1 ml each of working citrate and tartrate-citrate substrates for citrate blank, tartrate-citrate and sample tubes. Place the tubes in a water bath at 37° C for 5 minutes. Add test serum of 0.2 ml to the sample tubes. Mix the contents by swirling the tubes rapidly and replace in the water bath. Incubated the tubes at 37° C for 30 minutes followed by the addition of 4 ml of 0.1 N NaOH. Remove the tubes from the water bath, and add 0.2 ml of double distilled water to the citrate-substrate blank tube and tartrate-citrate blank tube. Mix the contents and read the absorbance at 410 nm Treatp-nitrophenol as a standard solution with a concentration of (0.04 mmol/l) in the similar way and express serum tartrate resistant acid phosphatase as U/l.

Estimation of serum albumin

Serum albumin was estimated by the method of Reinhold (1953). To serum (0.4 ml) and 6 ml of 27.8% of sulphate-sulphite solution. Mix the contents by inverting the tube. Add 3 ml of ether to the serum sulphate-sulphite mixture, put the stopper and mix the contents well. Centrifuge the tubes for 5 min at 2,000 rpm. Pipette out (2 ml) the clear solution and add 5 ml of the biuret reagent in a test tube. For serum blank, add 2 ml of the serum sulphate-sulphite mixture to 5 ml of the tartrate-iodide solution and mix. For biuret blank, add 2 ml of the sulphate-sulphite mixture to 5 ml of the biuret reagent and mix. Mix the contents and place the tubes in a water bath 37° C for 10 minutes. Allow the contents to

cool for five minutes at room temperature, and then read the absorbance at 555 nm. Treat bovine albumin as a standard protein solution with different concentrations (1 mg/ml) in the same way and express serum protein as mg/ml. and express serum albumin as g/dl.

Statistical analysis

Statistical analysis of the data was also performed using a two-way ANOVA

calculation with Duncan's pair wise comparisons between groups.

RESULTS AND DISCUSSION

Serum parameters such as calcium, phosphorus, alkaline phosphatase, tartrate resistant acid phosphatase, creatinine and albumin between males and females epileptic and non- epileptic groups are presented in Table 1 and 2.

Table 1: Bone mineral status between males and females in epileptic group

Serum Parameters	Epileptic group							
	Age Group <14 years		Age Group 15-18 years		Age Group 19-50 years		Age Group >50 years	
	Males (n=13)	Females (n=10)	Males (n=19)	Females (n=09)	Males (n=60)	Females (n=45)	Males (n=10)	Females (n=10)
Calcium(mg/dl)	9.95 ± 0.14*	9.57 ± 0.13	9.71 ± 0.15	9.77 ± 0.08	9.8 ± 0.06**	9.58 ± 0.06	9.92 ± 0.16**	9.37 ± 0.08
Phosphorus(mg/dl)	4.3 ± 0.2	4.56 ± 0.35	3.61 ± 0.17	4.09 ± 0.23	3.93 ± 0.10*	3.64 ± 0.09	4.49 ± 0.14	3.34 ± 0.11**
Alkaline Phosphatase (IU/l)	122 ± 8.22	168.4 ± 18.98*	173.41 ± 21.46**	89.11 ± 12.93	89.11 ± 12.93	98.11 ± 7.82*	75.2 ± 9.71	106.61 ± 10.86*
Tartrateresistant acid phosphatase (U/l)	10.75 ± 1.36	17.36 ± 3.18*	10.04 ± 0.9	13.94 ± 2.15*	9.46 ± 0.86	12.27 ± 1.14*	8.66 ± 0.88	14.62 ± 0.95**
Creatinine (g/dl)	0.66 ± 0.03	0.68 ± 0.04	0.89 ± 0.09**	0.57 ± 0.07	0.95 ± 0.04**	0.76 ± 0.02	1.41 ± 0.28	1.16 ± 0.33
Albumin(mg/dl)	3.97 ± 0.09	4.11 ± 0.09	3.94 ± 0.06	3.92 ± 0.06	3.86 ± 0.04	3.75 ± 0.05	3.5 ± 0.13	4 ± 0.13

All values are Mean ± SE; 95% CIs in parentheses; P * <0.05 **<0.01 (Significance between the males and females of same age in epileptic group)

Table 2: Bone mineral status between males and females in non-epileptic group

Serum parameters	Non-Epileptic group							
	Age Group <14 years		Age Group 15-18 years		Age Group 19-50 years		Age Group >50 years	
	Males (n=16)	Females (n=17)	Males (n=15)	Females (n=14)	Males (n=46)	Females (n=85)	Males (n=29)	Females (n=21)
Calcium (mg/dl)	10.33 ± 0.13	9.99 ± 0.17	10.25 ± 0.17	10.15 ± 0.19	10.32 ± 0.11	10.16 ± 0.07	10.2 ± 0.12	10.31 ± 0.15
Phosphorus (mg/dl)	3.08 ± 0.13	3.01 ± 0.11	3.1 ± 0.16	3.16 ± 0.08	3.12 ± 0.08	3.05 ± 0.05	2.89 ± 0.06	3.02 ± 0.08
AlkalinePhosphatase (IU/l)	61.2 ± 2.58	56.41 ± 4.78	56.95 ± 6.47	50.34 ± 5.68	46.85 ± 2.85	53.87 ± 2.17*	51.26 ± 3.49	63.41 ± 4.66*
Tartrateresistant acid phosphatase (U/l)	7.42 ± 1	7.2 ± 0.99	5.66 ± 0.56	7.41 ± 0.42*	6.87 ± 0.43	7.3 ± 0.43	6.34 ± 0.45	7.88 ± 0.64*
Creatinine (g/dl)	0.81 ± 0.05**	0.5 ± 0.06	0.68 ± 0.07	0.52 ± 0.08	0.65 ± 0.04	0.77 ± 0.03*	0.83 ± 0.03	0.85 ± 0.04
Albumin(mg/dl)	4.46 ± 0.07	4.92 ± 0.08**	4.4 ± 0.12	4.42 ± 0.08	4.54 ± 0.06**	4.17 ± 0.05	4.66 ± 0.08**	4.08 ± 0.09

All values are Mean ± SE; 95% CIs in parentheses; P * <0.05 **<0.01 (Significance between the males and females of same age in non-epileptic group)

Table 3: Correlations between serum biochemical parameters of epileptic group

Serum Parameters	Serum Parameters	r-value
Calcium and	Creatinine	0.0152*
	Tartrate resistant acid phosphatase (TRACP)	-0.155*
	Albumin	0.316**
Alkaline phosphatase and	Creatinine	-0.178*
Creatinine and	Albumin	-0.155*

P * <0.05; ** <0.01

Table 4: Correlations between serum biochemical parameter of non-epileptic group

Serum Parameters	Serum Parameters	r-value
Calcium and	Phosphorus	0.144*
Alkaline phosphatase and	Tartrate resistant acid phosphatase (TRACP)	0.131*

P * <0.05

Calcium is essential in maintaining bone health and plays an integral role in the homeostasis mechanism that takes place between blood and bone calcium levels

Mahan and Krause's, 2000; Sander and Emery, 2003). The mean serum calcium is higher in epileptic males of all age groups except in 15-18 yrs age group compared to females. The differences found are significant at $p < 0.05$ in < 14 yrs age group and $p < 0.01$ in 19-50 and > 50 yrs age groups. In non-epileptic males the levels of the serum calcium are higher compared to females except in > 50 yrs age group. No significant difference was observed between males and females in any age group. The serum calcium levels in both the sex groups of epileptic and non-epileptic groups are found to be within the normal range of 9.0 to 11.5 mg/dl (Varley, 1988).

The mean serum phosphorus is higher in epileptic males of < 14 yrs and 15-18 yrs age groups compared to females. The differences found are significant at $p < 0.05$ in 19-50 yrs and at $p < 0.01$ in > 50 yrs age groups. In non-epileptic males the levels of the serum phosphorus are higher compared to females in < 14 yrs and 19-50 yrs age groups. No significant difference is observed between males and females of any age group. The mean of all groups are found to be within the normal range of 2.5 to 4.5 mg/dl (Varley, 1988).

Calcium and phosphorus levels have been reported to be within normal ranges during treatment with anti-epileptic drugs (Kruse et al., 1977; Keck et al., 1982; Ala-Houhala et al., 1986; Ohoe et al., 1988; Deda et al., 1993). However, other studies did not show the similar results (Crosley 1975; Tjellesen et al., 1983). Frequency of decrease in calcium level by anti-epileptic drug usage between 3-30% has been reported (Hahn et al., 1972; Bouillon et al., 1975; Hahn et al., 1978; Schmitt et al., 1984).

Although calcium levels of epileptic group are within the normal range, the bone turnover is high. The dietary calcium of epileptic group is low and the dietary phytates is high which in turn hinders the absorption of dietary calcium leading to low serum calcium levels. To maintain the

serum calcium levels, PTH acts on the bone and mobilizes calcium from bone to the blood circulation. This condition leads to secondary hyperparathyroidism. The increase in bone resorption is clearly shown by increase in serum TRACP and serum alkaline phosphatase levels.

Alkaline phosphatase is the most frequently used biochemical marker of bone formation, and increases have been documented in adults and children receiving anti-epileptic drugs (Gough et al., 1986; Valimaki et al., 1994; O'Hare et al., 1980). The mean serum alkaline phosphatase is higher in epileptic females of < 14 , 19-50 and > 50 yrs age groups compared to males. The differences found are significant ($p < 0.05$) in < 14 , 19-50 and > 50 yrs age group and at $p < 0.01$ in 15-18 yrs age group. In non-epileptic males the levels of the serum alkaline phosphatase levels are high compared to females in < 14 yrs and 15-18 yrs age groups. The differences found are significant ($p < 0.05$) in 19-50 and > 50 yrs age groups. These values of non-epileptic males and females of all age groups are found to be within the normal range of 23 to 92 IU/l (Varley, 1988). In epileptic group, males in < 14 and 15-18 yrs age groups, females in < 14 , 19-50 and > 50 yrs age group had high serum alkaline phosphatase levels than reference range.

Normal calcium levels in all respondents and a significant increase in serum alkaline phosphatase activity in epileptic subjects is an indicator of biochemical abnormality of bone mineral homeostasis. It has been reported that ALP elevation in children receiving anti-epileptic drugs was not indicative but a warning for metabolic bone diseases and rickets (Kruse et al., 1977; Deda et al., 1993). Studies in ambulatory children and adults, consistently demonstrate significant increase in bone alkaline phosphatase, particularly with phenytoin and reduction in serum calcium in patients on enzyme-inducing AED compared with controls (Tolman et al., 1975; Hunt et al., 1986; Keck et al., 1982;

Pack et al., 2005; Kumandas et al., 2006; Mintzer et al., 2006). Other markers of bone turnover also appear to be consistently elevated in patients on AEDs include both enzyme and non-inducing (Valimaki et al., 1994; Erbayatet al., 2000; Sato et al., 2001; Verrotti et al., 2002).

Tartrate resistant acid phosphatase is osteoblast specific enzyme and acts as a significant bone resorption marker (Minkin, 1982; Halleen et al., 1999; Halleen et al., 2000). The mean serum TRACP is higher in females of all age groups in both epileptic and non-epileptic groups, except in non-epileptic females <14 yrs age group. In epileptics, significant differences found at 5% level ($p < 0.05$) in <14, 15-18 and 19-50 yrs age groups and at $p < 0.01$ in >50 age group. In non-epileptics, significant difference ($p < 0.05$) is found in 15-18 and >50 yrs age groups. The normal ranges for serum TRACP are 2.3 to 7.5 U/L for men and 0.3 to 8.2 U/L for women. The values of males and females of epileptic group are higher than the respective normal ranges. This shows the bone turnover is high in both males and females of the epileptic group. Females of epileptic groups show higher levels of TRACP over males of the same group. The higher levels of TRACP in females indicate the higher rate of resorption over males which leads to osteopenia and then to osteoporosis. Also, calcium is negatively and alkaline phosphatase is positively correlated with TRACP levels. This is the evidence for increase in bone turnover in epileptic group. Creatinine is the breakdown product of creatine, it is released in to the blood and filtered by the kidneys and is a biochemical marker to know the kidney functioning (Yuegang et al. 2008). In epileptic group, males exhibited high serum creatinine levels compared to females except in <14 yrs age group. Significant differences are observed in the age groups of 15-18 and 19-50 yrs $p < 0.01$. In non-epileptic group, males have higher serum creatinine levels compared to females in the age groups <14 and 15-18

yrs. Significant difference are observed in the age groups <14 yrs at 1% level and in 19-50 yrs at 5% level. The values of males and females of epileptic and non-epileptic groups are found to be within the normal range of 1-2 mg/dl (Varley, 1988).

In epileptic group, males have high serum albumin levels compared to females except in <14 and >50 yrs age groups. No significant differences are observed between males and females of all age groups. In non-epileptic group, males have high serum albumin levels compared to females except in <14 age groups. Significant ($p < 0.01$) differences have been observed between males and females in <14, 19-50 and >50 yrs age groups. The values of males and females of epileptic and non-epileptic groups are found to be within the normal range of 3.7 to 5.3 g/dl (Tietz, 2005).

Correlation between serum biochemical parameters of epileptic and non-epileptic groups is presented in Table 3 and 4. In epileptic group, correlations between serum calcium and creatinine; serum calcium and albumin are found to be positive and significant at $p < 0.05$ and $p < 0.01$ levels, respectively. Correlations between serum calcium and TRACP; serum alkaline phosphatase and creatinine; serum creatinine and albumin are found to be negative and significant at $p < 0.05$ level. In non-epileptic group, correlations between serum calcium and serum phosphorus; serum alkaline phosphatase and TRACP are found to be positive and significant at $p < 0.05$.

CONCLUSIONS

Serum calcium, phosphorus, creatinine, protein and albumin levels of epileptics and non-epileptics was observed to be in the normal range. A significant increase in serum tartrate resistant acid phosphatase and alkaline phosphatase levels in epileptic males and females is an indicator of biochemical abnormality of bone mineral homeostasis.

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