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STUDY OF URINARY HYDROXYPROLINE, SERUM PSEUDO CHOLINE ESTERASE, SERUM PROTEIN ELECTROPHORESIS ABNORMALITIES IN PEM CHILDREN

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ABSTRACT

Introduction: Hydroxyproline is a translation modification of amino acid for the formation of collagen in PEM (Protein energy malnutrition) children the hydroxylproline is excreted through urine not seen.The pseudo choline esterase lowers in the PEM children because of liner enlarged and impaired LFT and at the same time, the hypoalbuminemia is observed by an electrophoretic pattern of A: G ratio. ***Materials and Methods:*** This case-control study was carried out in the Index Medical College, Department of Pediatrics. This study population consisted of 75 cases of PEM and 50 controls. The data and complete clinical history of the subjects wascollected in association with the departments of Social and Preventive medicine and Pediatrics. The analysis of urinary hydroxyproline, serum pseudo choline esterase and A: G ratio of proteins by electrophoretic pattern was done. ***Results:*** In PEM childrenthe analyzed parameters are significantly lowered compared tonormal healthy children these parameters observed from PEM children are useful for the control of the mortality and morbidity rate in children. ***Conclusion:*** This study mostly says that nutritional dwarfing of protein deficiency calories is seen in this condition. The clinical finding of electrophoretic pattern protein separation is very low bands appear the band are densitized by densitometry.

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INTRODUCTION

In 1932 protein-energy malnutrition was first described by Dr.Cieely Williams PEM is a deficiency disease caused in children due to a food gap between the intake and essential requirement of dietary factors as an RDA. It affects the poor underprivileged communities in these communities with clinical signs and symptoms of poor weight gain slowing of linear growth, edematous, and severe muscle wasting caused by protein caloric malnutrition.[2] In this condition mostly the PEM children affect a lot of metabolic changes the changes we are follow these children to asses the hydroxyproline in urine.[5] Proline is a nonessential amino acid whenit undergoes post-translation modification it turns the hydroxyproline.[3,6] it is a key molecule for the formation of collagen, it is a connective tissue. Hydroxyl proline is also important for bone formation in the bone matrix as a key molecule in osteocalcin protein.[7]The hydroxyproline is excreted through normal healthy children because collagen gets elasticity and bone resorption of collagen leads to hydroxyproline in urine but PEM children have poor hydroxyproline in urine.

Pseudocholine esterase is a glycoprotein enzyme that is synthesized in the liner and enters into blood circulation.[8] It is important for the hydrolysis of the exogenous and circulatory choline esterase.[9] Choline esterase is a key enzyme for the metabolism of acetylcholinefor the hydrolysis of acetate and choline splitting of the covalent form of acetylcholine.[10] The enzyme inhibits the acetylcholine concentration in the synaptic cleft. In case of excess accumulation of acetylcholine in CNS which can result in fatal convulsions will occur.[11,14] In PEM children dysfunction of the liver and lack of protein leads to impair the synthesis of pseudocholine estrase.[12,13] Serum proteins represented mainly albumin, globulins, and fibrinogen these proteins are distributed in blood for the regulation of various physiological and biochemical functions.[15,16] In PEM children serum protein electrophoretic pattern is abnormal as compared to normal healthy children.[18,19]Dysproteinemia develops in malnutrition conditions the electrophoretic serum protein estimation in PEM is an important prognostic indicator that is associated with increased mortality and morbidity.[4,17,20,21]

MATERIALS AND METHODS

The study was done on 100 children (subjects) suffering from PEM and 50 normal healthy children (controls). All children who either came to the outpatient department or were admitted to the pediatric ward of Index Medical College and Research Center that satisfied the case definition criteria of PEM were included in the study. (IAP classification)[31]

Inclusion Criteria

Cases: All children who either came to the outpatient department or were admitted to the pediatric ward of Narayana Medical College and Hospital and satisfied the case definition criteria of PEM were included in the study.

- Only children aged up to 12 years were included. All children with weight less than that expected for the age. (The term expected, weight for age was the 50th percentile of the Harvard Growth Standards according to the " Nutrition Sub Committee of the Indian Academy Of Pediatrics") were studied.
- Children of both sexes were included in the study.

Controls: Normal Healthy Children age upto 12 years were included.

Exclusion Criteria

- Children with lesions like lymphoma, tuberculosis, leukemia, or any other systemic disease leading to weight loss were excluded from the study.
- Children with dehydration, clinical evidence of infections or septicemia, nephrosis, liver cirrhosis, cardiac failure, and severe anemia have also been excluded from the study.

Parameters	Method
1) Weight	Weighing machine
2) Serum pseudocholinesterase	Kinetic Method [22,23]
3) Serum protein electrophoresis	Serum Protein Electrophoresis Kit [119]
4) Urinary hydroxyproline	Modified Neuman Logan Method [120]

Specimen Collection

Serum sample: About 5 ml of blood sample was collected from selected children under aseptic conditions from a vein. Then the sample was transferred to plain tubes to get serum. The samples were centrifuged and the serum thus obtained was either analyzed or stored at 2- 8 ºC.

Urine sample: 6 hourly urine samples were collected after giving proper instructions to children and parents. The urine sample was collected in a container containing 4 % thymol as a preservative for up to 6 hours. The 6-hour sample was thoroughly mixed following which 1 ml of urine sample was taken from this mixture for evaluation of hydroxyproline.

Weight: Weight is a sensitive indicator of nutritional status as acute nutritional deficiency can also be manifested by changes in weight in comparison to height which shows changes in case of chronic nutritional deprivation only.

Method: Weight should be measured correctly. For infants electronic weighing machine is ideal. For children above 1 year of age weighing machines with horizontal scales are reasonably good. Weight should be taken with the minimum possible clothing and should be plotted on a growth chart periodically. All cases and controls were subjected to anthropometric measurements. The expected weight for age was 50th percentile of the Harvard growth standards according to, "The nutrition sub-committee of the Indian Academy of Pediatrics".[27]

Serum Pseudocholinesterase. [23]

Method: Butyryl thiocholine potassium hexacyanoferrate III Method (Kinetic method)

Principle: Butyrylthiocholine is hydrolyzed by cholinesterase (CHE) to produce theodolite and butyric acid in the presence of potassium hexacyanoferrate (III), the absorbance decrease being proportional to the cholinesterase activity of the sample. The catalytic concentration is determined from the rate of decrease of hexacyanoferrate (III) measured at 405 nm, by means of the following reaction.

$$\text{Butyrylthiocholine} + H_2O \xrightarrow{\text{CHE}} \text{Thiocholine} + \text{Butyric acid}$$

$$2\ \text{Thiocholine} + 2OH + 2\ \text{Hexacyanoferrate III} \rightarrow \text{Dithiobis (choline)} + 2\ \text{Hexacyanoferrate II} + H_2O$$

The atypical isoenzyme of cholinesterase (AA) can be estimated through the "dibucaine number" indicating the inhibition of enzyme activity in the presence of dibucaine and is expressed in percentage.

Contents and Composition

1. Reagent I - Buffer Reagent

Pyrrophosphate	95 mmol/ l,
Hexacyanofolate (III)	2.5 mmol/l

2. Reagent II –

Butyrylthiocholine iodide reagent (pH 7.6)	60 mmol/l

Sample: Serum, Heparin, or EDTA Plasma.

Stability in Serum: Pseudocholinesterase is stable for 14 days in serum or plasma at 2- 8 ºC

Storage: Reagents can be stored at 2 – 8 ºC for 7 days. Reagents are stable until the expiry date shown on the label if the bottle is tightly closed with negligible contamination during their use.

Indications of deterioration of reagents: Presence of particulate material and turbidity in the reagents and if the absorbance of the blank is below 1.300 at 405 nm (1 cm cuvette).

Auxiliary reagents: Reagent (cod 11578): Dibucaine 0.3 mmol/l, after reconstitution 10 x 15 ml.

Reagent preparation- Mix gently 4 parts of Reagent I and 1 part of Reagent II.

Additional Requirement

- Analyzer- spectrophotometer or photometer with cell holder
- Cuvettes with 1 cm light path.

Automated Parameters

Wavelength	405 nm
Cuvette	1 cm light path
Temperature	37ºC
Measurement	Against water
Sample / Reagent	1: 67
Reaction	Kinetic
Reaction Direction	Decreasing
Delay/ Lag/Time	60 sec
Interval Time	30 sec
No. of Readings	03
Factor	62000
Blank Absorbance Limit	0.6
Low Normal at 37ºC	4850 U/I
High Normal at 37ºC	12000 U/I
Linearity	12000 U/I

Procedure:

Pipetting Scheme

Pipette into cuvettes	Blank	Standard	Test
Distilled water	15 µl	----	---
STD	---	15 µl	----
Sample	----	----	15 µl
Reagent	1000µl	1000µl	1000µl

Mix well and wait for 1 minute. Measure absorbance decrease after 30,60 and 90 seconds. Determine the ▲A/ minute.

Calculation and linearity: ▲A/ minute x 62000= U/L Cholinesterase.

This method is linear up to 12000 U/l

Reference Values

At 37ºC. Male 4620 – 11500 U/l
Female 3930 – 10800 U/l.

Quality control: Each lab should establish its own internal quality control scheme and procedures for corrective action if control doesn't recover within acceptable tolerances.[24]

Serum Protein Electrophoresis-[26](SPE)

METHODOLOGY

The paragon Serum Protein Electrophoresis (SPE) kit is intended for the electrophoretic separation of proteins in human serum, cerebrospinal fluid, and urine.Agarose gel electrophoresis has been used to separate human serum proteins into five generally distinct, well-resolved zones, which are composed of many individual proteins. The principle of electrophoresis is based on the fact that proteins, when placed in an electrical field, will migrate toward one of the electrode poles. The Paragon SPE kit provides for the electrophoretic separation of proteins in a buffered agarose gel. After electrophoresis, the proteins in the gel are immobilized in a fixative solution and the gel is dried to a film. The protein pattern is visualized by staining the film with a protein-specific stain. This pattern may be visually interpreted or quantitated by densitometry.[25]

Contents

Components	Quantity
SPE GELS	10
B-2 Barbital Buffer	1 Bottle
Paragon Blue Stain	1 Bottle
Templates, Blue	10
Template blotters	10
Gel Blotters	20
Instruction Sheet	1

Reactive Ingredients:

a) ***SPE GEL:*** *1*.0 % Agarose, 1.2% Barbital Buffer, >0.1% w/w Sodium azide, and non-reactive ingredients necessary for optimum performance.
b) B- 2 Barbital buffer, 18.2 g: 5,5- Diethylbarbituric acid, 10 mmol/l reconstituted.: 5,5- Diethylbarbituric acid Sodium salt, 50 mmol reconstituted.
c) Paragon Blue Stain, 5g: 8- amino- 7- (3- nitrophenyl azo) – 2- (phenyl azo)-1- naphthol- 3,6- sulfonic acid disodium salt, 0.5 %, reconstituted.

Reagent preparation, storage, and stability

1) SPE gels: Just prior to use, carefully remove a Gel from the foil package. Gels should be stored at room temperature, 18- 26 ºC, until expiry occurs. Improper temperature or storage conditions can result in atypical migration. Do not refrigerate or freeze.

2) B-2 Barbital buffer, pH 8.6 (0.05 ionic strength): Dissolve the contents of the buffer bottle into 1500 ml of deionized water. The unopened buffer should be stored at room temperature, 18- 26 ºC until expiry occurs. Reconstituted buffer stored in a closed container at room temperature is stable for 60 days or till the expiry date, if sooner.

3) Acid-alcohol solution, 20 % acetic Acid- 30 % methanol: To 1000 ml of deionized water, add 600 ml of methanol and 400 ml of glacial acetic acid. Mix properly. The solution should be stored in a closed container at room temperature, 18- 26 ºC.

4) Acetic Acid Solution, 5 %: To 2850 ml of deionized water, add 150 ml of glacial acetic acid. Mix nicely.

5) Paragon Blue stain, 0.5 %: Dissolve the contents of the stain bottle into 1 l of 5 % acetic acid solution. Mix nicely.

Specimen Collection: Biological fluid samples should be collected in the manner normally used for laboratory tests. Fresh-drawn serum is better, but samples stored at 2- 8 ºC for up to 72 hours may also be used. Hemolyzed or lipemic samples are not recommended for analysis.

Specimen preparation of serum specimens: For use with a paragon sample applicator, dilute all serum samples and controls with B- 2 Barbital Buffer by mixing 1 part sample with 4 parts buffer. Sample solutions should be freshly prepared on the day of use.

Electrophoretic procedure[29]

1) Prepare all samples and controls.
2) Fill each compartment of the electrophoresis cell with 45 ml of B-2 Barbital Buffer.

Acid-Alcohol Solution, I	300 ml
Paragon Blue Stain Solution	300 ml
Acetic Acid Solution, 5 %, I	300 ml
Acid- Alcohol Solution, II	300 ml
Acetic Acid Solution, 5 %, II	300 ml

3) Fill containers of wet processor with 300ml of the following solutions.
4) Remove SPE gels from the foil package and place them on a paper towel. Blot gently with a gel blotter. Discard blotter.
5) Template application:
   a) Bend the template lengthwise.
   b) Apply a template to the gel such that template slots contact the gel surface first.
c) Gently rub your finger across the template to ensure a seal.
6) When using Paragon sample applicator, apply 3-5 µ litre of the diluted sample across each template slot. Allow 5 min for diffusion after the last sample has been applied. Then gently blot the template with a template blotter. Discard blotter and template.
7) When using the Paragon multirange pipet, apply 0.5 µ liter of undiluted serum sample to the center of each template slot. Allow 2 minutes for complete diffusion after the last sample has been applied. Then gently remove the template and discard.
8) Place gel on the gel bridge assembly, aligning the + and - ve sides of the gel with corresponding positions marked on the gel bridge assembly. Place assembly into Paragon electrophoresis cell and cover cell.
9) Insert the Paragon electrophoresis cell into the power supply. Set the voltage to 100 V, turn the power on and electrophorese for 25 minutes.
10) Upon completion of electrophoresis, remove the gel from Paragon Electrophoresis Cell, and place it into the gel frame.
11) Place gel into Acid-Alcohol Solution I for 1-3 minutes.

12) Remove the gel from Acid-Alcohol Solution I . Wipe excess solution from the back of the gel and place in the Paragon dryer until completely dry.
13) Process dried gel in the following sequence:

Paragon Blue Stain	3 min
Acetic Acid Solution I	2 min
Acid-Alcohol Solution II	2 min
Acetic – Acid Solution II	2 min.

14) Remove gel from Acetic Acid Solution II.
15) Remove the gel from the gel frame.
16) Blot gel with gel blotter and place in paragon dryer until completely dry.
17) Evaluate the gel visually or scan with a suitable densitometer at 600 nm.

Quality Control: It is recommended that pooled sera or commercially available quality control sera be included in each electrophoretic procedure.[28] The serum protein pattern may be visually interpreted by comparing the sample percentage of each protein zone may be calculated. In either method, the presence of extra serum components or an increase or absence of normally occurring serum components indicates the necessity for further protein analyses.

Test Limitations

1. Hemolyzed samples can increase global values.
2. Gels not stored in a horizontal position may produce atypical electrophoretic patterns.
3. Light patterns may occur if the 0.5 µl sample is not applied to the center of the Template slot.
4. Inadequate wiping of the Paragon Multirange pipet tip after rinsing may cause sample dilution and result in lighter electrophoretic patterns.
5. Lipemic samples are not recommended for analysis.

Expected values: The following expected normal range values for the Paragon SPE kit were determined by calculating the mean values ±2 standard deviations for each protein fraction from a population of 60 apparently healthy male and female adults from southern California.[27]

Protein Fraction	Relative Percentage (%)		
Albumin	55	to	65 %
Alpha 1	2	to	4 %
Alpha 2	6	to	12 %
Beta	8	to	12 %
Gamma	12	to	22 %

Urinary hydroxyproline (modified Neuman Logan)[29]: For estimation of urinary Hydroxyproline 6 hourly urine collection was done after adding a preservative (HCl: 5ml / l of urine)

Method- Modified Neuman and Logan Method.

Principle: Hydroxyproline is treated with $Cuso_4$& H_2O_2 in an alkaline solution; this results in the formation of ▲Pyrroline – 4- carboxylic acid, which upon acidification is converted to pyrrole – 2 – carboxylic acid. The latter condenses with p-dimethylaminobenzaldehyde to give the colored complex which is measured at 540nm.[30]

Reagents:

Reagents:

1. Copper sulfate ($CuSO_4$) (0.01 M):Dissolve 0.159g of $Cuso_4$ in 100ml D/W
2. Sodium hydroxide (NaOH) (2.5N): Dissolve 10g of NaOH in 100ml D/W
3. 6% Hydrogen Peroxide (H_2O_2
4. Sulphuric acid (H_2SO_4) : (3N)
5. P-dimethyl amino benzaldehyde : (5% solution in n-propanol)
6. Hydroxyproline standards : They are prepared corresponding to 5, 10, 20,25,30,35,40,45, g of OH proline.

Procedure:

Reagents	Blank(B)	Standard(S)	Test (T)
Urine	--	--	1ml
Std	--	1ml	--
Distilled H_2O	1m	--	--
$CuSO_4$ (0.01M)	1ml	1ml	1ml
NaOH (2.5N)	1ml	1ml	1ml
6% H_2O_2	1ml	1ml	1ml
H_2SO_4 (3N)	1ml	1ml	1ml
P- dimethyl amino benzaldehyde	2ml	2ml	2ml

Mix and wait for 5 minutes. Place in a water bath at 80ºC for 5 minutes. Cool in ice followed by keeping in a water bath at 70ºC for 16 minutes. Color is read at 540nm.

Calculation: The concentration of hydroxyproline in the samples was read directly from the std curve.

Reference values in children-

0 – 3 days	8 – 20 mg/day
4 days - 11 months	32 – 63 mg/day
1 – 10 Years	15 – 150 mg/day
11 – 14 yrs	68 – 169 mg/day

RESULTS

The comparison of urine proline hydrolise between malnourished cases and control of normal healthy children had shown that significantly higher values are observed in PEM children. Table 1. Shows the comparison of hydroxyl proline between case and controls the urine hydroxyl proline significantly high in the cases then controls pvalue 0.000

Table 1. Urinary Hydroxyproline

Parameter	Cases	Controls	t value	p-value
Urinary hydroxyproline (mg/day)	14.046 ± 8.34	46.8 ±25.7	8.012	0.000

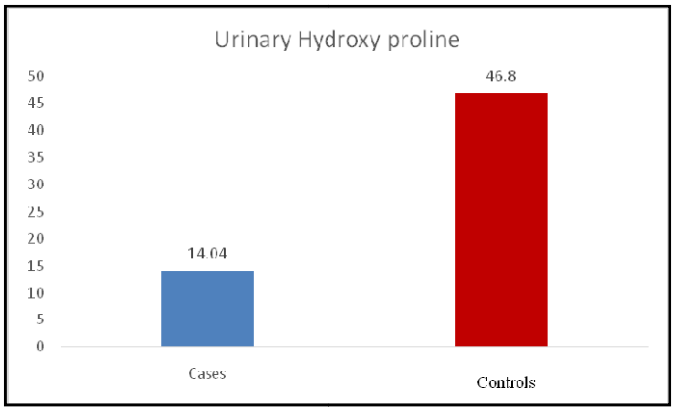

Figure 1: Shows the comparison of urinary hydroxyl proline between cases and controls.The urine hydroxyl proline is significantly higher in the cases than the control mean value[33].

Table 2. Show the comparison of serum pseudo choline esterase between cases and controls the values of serum pseudocholinesterase were significantly lower in cases than in control as pvalue 0.000.

Table 2. Serum Pseudocholinesterase

Parameter	Cases	Controls	t value	p-value
Serum pseudocholinesterase (U/L)	2745 ± 857.56	6689.5±14 57.1	14. 355	0.000

Controls

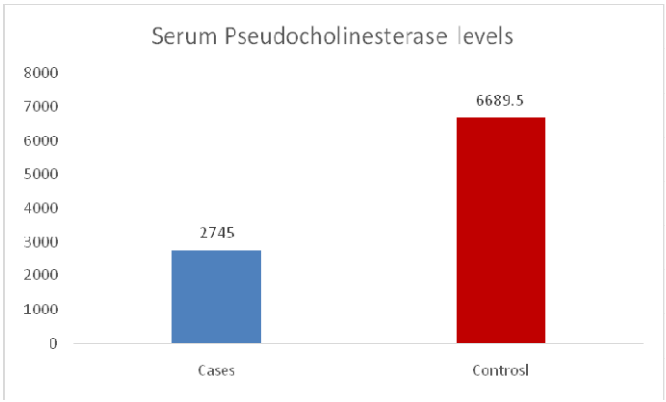

Figure 2 Shows the comparison of serum pseudocholine esterase between cases and controls the values of serum pseudocholinesterase were significantly lower in cases than in the control mean value[34].

Table 3: Shows the electrophoretic pattern of a fraction of protein bounds assed by densitometry values comparison between cases and controls. The values of serum protein significantly change and are observed compared to controls as p-value 0.000

Table 3. Serum Protein Electrophoresis

Different Fractions Of Serum Protein Electrophoresis (%)	Cases	Controls	t value	P value
Albumin	41.3± 5.2	59.8 ± 3.1	10. 070	0.000
alpha 1 globulin	7.02± 2.44	3.8 ± 1.0	6.765	0.000
alpha 2 globulin	12.1 ± 3.89	9.9 ± 2.7	3.065	0.004
Gamma globulin	27.3 ± 5.31	17.9 ± 3.7	7.571	0.000
beta globulin	6.54 ± 1.64	9.9 ± 0.9	7.982	0.000

Controls

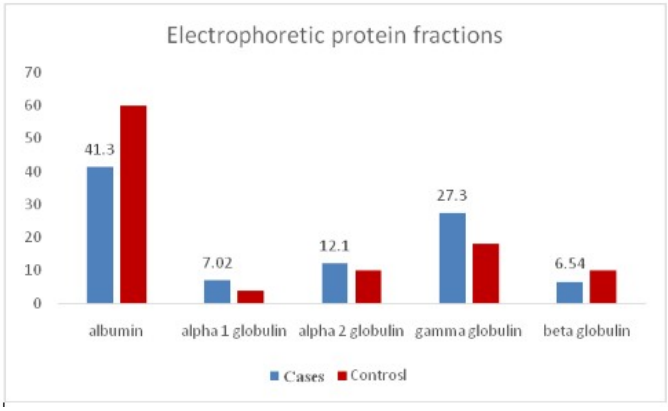

Figure 3: shows the electrophoretic pattern of a fraction of protein bounds assed by densitometry values comparison between cases and controls. The values of serum protein significantly change and are observed compared to the control mean value[35].

DISCUSSION

The comparison of urine hydroxyproline levels between malnourished case and healthy children showed a significant reduction in cases than controls as shown in Table 1. The mean urine hydroxyproline value for malnourished children was14.046 while that control was 46.8 (Figure 1) the difference was highly significant statistically as p-value was <0.000. Therefore, the urine hydroxyproline is a good marker for assessment of the rate of collagen elasticity and bone resorption is impaired in PEM children when lack of protein diet.Finally, hydroxyl proteinuria of the study was proposed it be the most sensitive index of PEM children because of its rapid response of refeeding of proteins and full chlorogenic diet priscribe. Table 2 shows the serum pseudo choline esterase in PEM children and healthy children whichindicates significantly lower values in PEM children than in controls. The mean value of serum pesudocholine esterase in PEM children was 2745, where as in controls it was 6689.5 (Figure 2).The difference is highly significantas p-value is <0.000.This finding of this study suggest serum pseudo choline esterase is significantly lowered in PEM children the finding of pesudocholine esterase synthesis lowered in PEM children because due to lack of insufficient protein diet and liner dysfunction when lack of essential diets factor the supplement of protein in growth required children. Table 3 shows the values compared to malnourished children and controls of normal healthy children shows the electrophoretic fraction of protein band in albumin is very low compared to controls with the mean valueAlbumin-41.359.8, alpha 1 globulin - 7.02, 3.8 , alpha 2 globulin- 12.1, 9.9, Gamma globulin-27.3, 17.9, beta globulin- 6.54, 9.9 statistically highly significant p0.000. It is a significant study for the protein status in PEM children compared to Normal Healthy children.Figure 3 shows the electrophoretic pattern of a fraction of protein bounds assed by densitometry values comparison between cases and controls. The values of serum protein significantly change and are observed compared to the control mean value[35]

CONCLUSION

When lack of protein diet and lack of caloric diet leads to bone resorption of osteoclast activity is enhanced this result hydroxyproline enters into blood circulation leading to excretion through urine and at the same time over collage elasticity leads to collagen resorption of bone toextreme happens in PEM children. It is also one of the factors for hydroxyl proteinuria significantly very low.It is a good diagnostic marker for the prevention of PEM mortality and morbidity as a cured history of PEM children we collect data on hypoproteinemia the electrophoretic pattern observed the mobility of protein fractions in the electrophoresis observed the albumin density is very low and the rest of the the the the globulin fractions are having high density except γ-globulins it indicates the hypoproteinemia accuracy assay the PEM children because of low protein diet and liver impairs the production of total protein. It is observed in grade 4 malnutrition children in Indore premises villages. Thisstudy mostly says that nutritional dwarfing of protein deficiency calories is seen inthis condition. The clinical finding of electrophoretic pattern protein separation is very very low bands appear the band are densitized by densitomety.The pesudocholine esterase is significantly lower in PEM children due to dysfunction of the liver for malnutrition condition insufficiency of protein and amino acids intake for the synthesis of various important functional proteins along with this pseudocholine esterase.The finding of pseudocholine esterase is also an important marker for the prevention of fatalconvulsions in PEM children. Finally the suggestion of this study significantly all markers are abnormal in PEM children in Indore primase villages.

Statements & Declarations

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Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Bokkisam Suneel, Jaya Jain, Desu Muralikrishna, Ashutosh Jain and Molluru Deepa. The first draft of the manuscript was written by Bokkisam Suneel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the

Institutional Ethics Committee, Index Medical College and Research Center, Malwanchal University, Indore.

Consent to participate: Written informed consent was obtained from the parents or guardians of children.

Consent to publish: The authors affirm that the parents of children included in this study provided informed consent for publication.

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