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Indian Journal of Forensic and Community Medicine

Journal homepage: https://www.ijfcm.org/

Original Research Article

Determination of postmortem interval and cause of death: Do the levels of biochemical parameters in vitreous humor provide an additional assistance to medico legal expert?

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ARTICLE INFO

Article history:
Received 20-11-2022
Accepted 26-12-2022
Available online 25-04-2023

Keywords:
Postmortem interval
Vitreous humor
Biochemical test
Sodium
Potassium calcium

ABSTRACT

Introduction: An upward rise has been observed in the pattern of unnatural deaths in the recent years and the same has been found during the post mortem examination. During the trial in the court of law prosecution as well as defense require the time passed death before examination and the cause of death in particular circumstances, as it is mandatory to fix the exact duration either to punish the guilty or NOT to punish the innocent.

Vitreous humor is different from other body fluids as it has relative stability in its composition so as also biochemical parameters. It was further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry and may be a useful aid in establishing postmortem diagnoses. Various studies have been carried out on vitreous electrolyte concentration changes to arrive at postmortem interval. Many workers found the importance of potassium in the determination of postmortem interval, whereas among all the biochemical parameters, vitreous potassium is found to be more useful to find out time since death. Postmortem biochemical analysis of vitreous humor may be helpful in the establishment of antemortem disease or metabolic disorder.

Materials and Methods: The present study was carried out jointly in department of Forensic Medicine and Toxicology and department of Biochemistry of Surat Municipal Institute of Medical Education & Research (SMIMER), Surat. A total of 100 subjects were taken up for this study. The samples were collected from dead bodies during postmortem examination with all aseptic precautions. A detailed performa was made with all the details of age, sex, length, weight and date and time of admission, date and time of death and manner of death such as suicide, homicide, sudden death and accidents was recorded. Cases below 15 years of age were not considered in this study.

Result: Out of 100 cases there were 24 (24%) females and 76 (76%) were males, out of these 56% were between 15-35 years, 32% were of 36-55 years, 9% were of 56-65 years, and 3% were of 66-77 years age group. According to the manner of death 50 subjects were of accident, 26 subjects were of sudden death, 21 subjects were of suicide and 3 subjects were of homicide. According to time interval between sample collection and time of death, 47 subjects were of 0-24 hours, 36 subjects were of 24-48 hours, 11 subjects were of 48-72 hours and 6 subjects were of 72-96 hours. In present study we observed that there is definitive correlation between time of death and changes in level of these biochemical parameters.

Conclusion: This was a preliminary study in this region and can be considered as a pilot study. The levels of vitreous Potassium have been found to be significantly altered as the time progresses after death. A progressive decrease in sodium/potassium and increase in potassium/ calcium ratios was also observed.

https://doi.org/10.18231/j.ijfcm.2023.007

1. Introduction

An upward rise has been observed in the pattern of unnatural deaths in the recent years and the same has been found during the post mortem examination. To find out the cause of death and postmortem interval/time since death during the post mortem examination, the onus is on the attending investigating officer. If the suspected death is either homicidal and/or due to suspicion the relevance of finding out exact cause of death becomes prominent and the responsibility of investigating officer increases tremendously. During the trial process prosecution as well as defense require the time of incidence, the time passed before examination and the cause of death in particular circumstances as it is mandatory to fix the exact duration either to punish the guilty or NOT to punish the innocent. Looking into these aspects' determination of cause of death and estimation of time since death attains a lot of importance for both investigators and law enforcement officers.

It was further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry and may be a useful aid in establishing postmortem diagnoses. Various studies have been carried out on vitreous electrolyte concentration changes to arrive at postmortem interval.[1] Many workers found the importance of potassium in the determination of postmortem interval, whereas among all the biochemical parameters, vitreous potassium is found to be more useful to find out time since death.[2,3]

Biochemical parameters are analysed using body fluids such as blood, cerebrospinal fluid, synovial fluid, pericardial fluid and vitreous humor in the diagnosis of various diseases. Blood gets haemolyzed after death hence blood/serum is unsuitable for analysis of many biochemical parameters. Vitreous humor is different from other body fluids as it has relative stability in its composition so as also biochemical parameters. Vitreous humour, located between the lens and the retina, filling the center of the eye and vitreous body is an extracellular matrix containing fibrillar structural proteins associated with varying amount of hyaluronic acid, glycoproteins and proteoglycans.[4] It was further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry and may be a useful aid in establishing postmortem diagnoses. Various studies have been carried out on vitreous electrolyte concentration changes to arrive at postmortem interval.[1] Many workers found the importance of potassium in the determination of postmortem interval, whereas among all the biochemical parameters, vitreous potassium is found to be more useful to find out time since death.[2,3] Because of its uniqueness, is preferred, as it is anatomically separated, resistant to putrefaction for a long time and hardly contaminated even in the late post mortem intervals. In other words it is the most sterile fluid available without the possibility of contamination. Looking into the characteristics of vitreous fluid, it can be considered as the most suitable body fluid for postmortem biochemical analysis for medicolegal purpose.

Biochemical analysis of vitreous humor will help in assessing the antemortem metabolic status of an individual and in predicting the antemortem serum biochemistry of an individual for diagnosis of various diseases resulting in changes in concentration of electrolytes and clinical chemistry parameters.[1] It was further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry and may be a useful aid in establishing postmortem diagnoses.[5]

Concentration of several analytes in vitreous humor reflects blood level immediately prior to death and are frequently used for the postmortem diagnosis of various pathological conditions. Glucose and acetone levels can aid in the post-mortem diagnosis of diabetes. Potassium concentration is used by some for the estimation of postmortem interval.[6] Sodium and chloride levels can disclose evidence of severe dehydration or water intoxication. Urea and creatinine can disclose evidence of renal failure.[7]

1.1. Electrolyte concentration

Vitreous humor is an inert, transparent, colorless, jelly like hydrophilic gel that serve the optical function and also act as an important supporting structure for eyeball. Vitreous cavity is bounded anteriorly by the lens and ciliary body and posteriorly by the retina. It weighs nearly 4g and occupies the volume of almost 4cc, which is approximate two-thirds volume of the entire globe. Vitreous is an extracellular material composed of approximately 99% of water.[8]

Vitreous electrolyte levels such as chloride, sodium, magnesium, calcium and potassium were reported to be useful in the postmortem judgement. Higher vitreous chloride concentration in posterior and anterior chamber than in plasma was reported by some studies.[8] Exchange of chloride ion seems to occur across both the retina and the posterior chamber leading increase in the concentration of chloride. Divergent findings were reported regarding the usefulness of concentration of sodium and magnesium in vitreous humour in the determination postmortem interval.[9] Farmer et al.[1] studied the levels of magnesium, potassium, sodium and calcium in postmortem vitreous humor from victims of fire fatalities and drowning and compared with human controls. They reported that the utility of magnesium in salt water drowning and sodium in fresh water drowning were related to the duration of the immersion, although the relationship was error prone. In a study of 50 diseased individuals Gregora et al.[10] did not find any utility of vitreous humour sodium and magnesium in the determination of postmortem interval.

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1.2. Diagnoses of other causes of death

A postmortem rise in the vitreous humor osmolality, independent of the postmortem solute status, has been suggested.[11] Utility of postmortem biochemistry in deaths due to uremia, dehydration and hyponatremia was reported.[12] Simultaneous elevation of vitreous humor sodium (> 155 mmol/L) and chloride (> 132 mmol/L) have been reported to reflect antemortem hydration and the renal status of a subjects.[3] To assess the antemortem renal status vitreous urea and creatinine concentration and in the diagnosis of hypercalcaemia, increased levels of vitreous humour calcium were observed to be useful. Additionally, detection of bilirubin in the vitreous humor has been reported to be always pathological, most likely indicating liver disease.[5]

1.3. Biochemical parameters and fixing of the time of death

In the forensic medicine, it is always a serious issue and dilemma to fix the time of death within the probabilities, in spite of its great importance. Traditional methods are only useful to give the approximate and inaccurate time since death. Various studies have been done on vitreous electrolyte concentration changes to arrive at postmortem interval. Many workers found the importance of potassium in the determination of postmortem interval, whereas among all the biochemical parameters, vitreous potassium is found to be more useful to find out time since death.

Many equations and corresponding formulae have been proved in literature to determine postmortem interval precisely. Sturner[6] developed an equation, Madea et al[13,14] revised a linear regression equation for postmortem interval. Farmers et al[1] reported an equation. Yogiraj[15] reported that both these formulas are only valid for adult individuals and cannot be established to PMI in the event of infant death and developed an equation for the calculation of postmortem interval using vitreous potassium.

Newman et al[16] studied to demonstrate rise in vitreous potassium value but did not attempt and correlate with postmortem interval. Jaffe[7] correlated the potassium in vitreous humor to the postmortem interval. Sturner[6] found a linear relation between the levels of potassium and postmortem interval. Coe[17] found a linear rise in potassium concentration during the postmortem interval of up to 100 hours.

2. Aim

Aim of the present study was to find out usefulness of postmortem biochemical analysis of vitreous humor in the establishment of antemortem disease or metabolic disorder. Parameters such as chloride, Potassium, calcium, glucose, urea and creatinine were included in this study. The utility of potassium and calcium and various ratios were examined to find out postmortem interval.

3. Objectives

1. To analyze Potassium, calcium, glucose, urea and creatinine in the vitreous fluid collected during post mortem examination in sudden death cases.
2. To assess the utility of vitreous levels of various biochemical parameters and different equations and formulae in the determination of postmortem interval and cause of death.

4. Materials and Methods

Present study was carried out by the department Forensic Medicine and Toxicology in collaboration with Biochemistry at tertiary care hospital after obtaining clearance from institutional ethical committee. A total of 100 subjects were included in this study. Vitreous samples were collected from dead bodies during postmortem examination observing all aseptic precautions. Parameters such as date and time of admission, date and time of death, manner of death such as suicide, homicide, sudden death and death due to accident and other parameters such as age, sex, length, and weight of the body was collected.

4.1. Inclusion criteria

Accident, suicide, homicide and sudden death cases were included in this study.

4.2. Exclusion criteria

Dead bodies having injuries over eyes and subjects below 15 years were not included in this study.

4.3. Anthropometric measurements

Body length was measured using a tape and weight of dead body was taken by putting it on a platform of electronic weighing machine.

Samples were collected by using 05 ml syringe with an 18-number needle in a clean sterile bulb. Needle was introduced in the eye through the outer canthus, 4.5 cm lateral to limbus and 5 ml of vitreous humor was aspirated as gradually as possible. Sample was immediately centrifuged for 10 minutes at 3000 revolutions per minute. Supernatant was used for biochemical analysis.

Laboratory investigations were carried out using ERBA-XL-300 fully auto analyzer and Stat fax-3300 semi auto analyzer. Estimation of serum sodium, potassium and chloride was carried out by ion selective method[18] using Combisys-II by Eschweiler, Germany. Standardization was carried out using appropriate calibrators and quality control samples

Serum creatinine and Urea were analyzed by modified Jaffe's reaction[19] and Berthelot methods[20] respectively

Fig. 1: The procedure to collect sample

Fig. 2: The procedure to collect sample

and calcium was analyzed by Arsenazo III (end point) method;[21] Farrel, 1984).[22] All the analyzers used in the analysis of the various biochemical parameters were calibrated as per the standard guidelines. Both appropriate and suitable controls and calibrators were used to maintain accuracy and precision. All the levels of controls were used to assess the accuracy of instrument as well as reagent systems. All data was collected and appropriate statistical were applied after doing test for normalcy. Nonparametric test was applied to those parameters not following the normalcy test and student t test to those with normalcy test.

The Table 1 shows that maximum number of subjects are in 15 – 35 years (52%) followed by 36 -55 years (32%), gender wise distribution, 73% male and 27% female.

Correlation of potassium concentration with PMI interval shows with increasing PMI there was an increasing trend in potassium levels.

Table 1: Distribution of subjects according to age and gender (n=100)

Age group	Male	Female	Number	Percentage
15 – 25 years	18	10	28	28%
26 – 35 years	19	09	28	28%
36 – 45 years	15	01	16	16%
46 – 55 years	12	04	16	16%
56 – 65 years	07	02	09	9%
66 – 77 years	02	01	03	3%
Total	73	27	100	100%

Table 2: Distribution of subjects based on manner of death (MOD) (n=100)

Manner of death	Number	Percentage
Suicide	21	21%
Homicide	03	03%
Accident	50	50%
Sudden death	26	26%
Total	100	100%

Table 3: Various biochemical parameters in vitreous fluid in the study subjects (n=100)

Parameter	Mean ± SD
Blood glucose (mg/dl)	75.68± 61.50
Urea (mg/dl)	30.97± 18.32
Creatinine(mg/dl)	1.47 ± 1.47
Calcium(mg/dl)	9.15 ± 10.07
Sodium(mmol/L)	137.59 ± 15.98
Potassium(mmol/L)	9.25 ± 2.71915
Chloride(mmol/L)	112.17 ± 13.83

5. Results

Table 1 shows that maximum number of subjects are in 15 – 35 years (52%) followed by 36 -55 years (32%) and gender wise distribution are 73% male and 27% female.

Table 2 shows manner of death - suicide (21), homicide (3), accident (50) and sudden death (26).

Table 3 various biochemical parameters like calcium, urea, creatinine, sodium, potassium and chloride were analyzed and presented.

Table 4 gives the concentration of various biochemical parameters in the subjects belonged to various age groups.

Table 5 gives concentration of urea, creatinine, calcium and chloride in the subjects in which death occurred due to different reasons.

Table 6 show correlation of Potassium concentration with PM interval. A correlation between potassium concentrations with PMI was observed. With increasing PMI there was an increasing trend in potassium levels.

6. Discussion

Analysis of biochemical parameters in blood, urine and CSF are not always suitable for postmortem chemical analysis

Table 4: Levels of vitreous humour biochemical parametersin different age groups (n=100) (Mean ± SD)

Age group	Number	Urea (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Sodium (mmol/l)	Potassium (mmol/l)	Chloride (mmol/l)
Group (1) 15-25	28	27.89 ± 15.95	1.52 ± 2.24	8.05 ± 1.16	134.32 ± 25.76	9.23 ± 2.68	112.67 ± 13.89
Group (2) 26-35	28	36.62 ± 24.47	1.53 ± 0.92	11.80 ± 18.56	139.27 ± 12.40	9.50 ± 2.59	112.93 ± 11.97
Group (3) 36-45	16	31.2 ± 15.09	1.36 ± 0.85	7.64 ± 0.73	137.86 ± 6.11	8.63 ± 1.59	115.46 ± 19.16
Group (4) 46-55	16	30.66 ± 13.18	1.92 ± 1.43	8.5 ± 1.56	138.93 ± 9.66	8.96 ± 2.37	110.86 ± 11.34
Group (5) >56	12	25.08 ± 12.71	0.82 ± 0.48	8.27 ± 1.55	138.83 ± 7.17	9.91 ± 4.32	106.75 ± 12.01
P- value		0.332	0.118	0.368	0.97	0.975	0.230

Value < 0 05 is significant

Table 5: Concentration of vitreous biochemical parameters in the subjects with different causes of death (n=100)

Manner of death	Number	Urea (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Chloride (mmol/l)
Group (1) Suicide	21	34.80 ± 22.33	1.70 ± 2.49	8.21 ± 1.20	113.47 ± 10.22
Group (2) Homicides	03	30.0 ± 2.82	1.03 ± 0.57	8.06 ± 0.89	137.33 ± 9.56
Group (3) Accident	50	30.28 ± 16.11	1.36 ± 0.89	10.19 ± 14.04	109.84 ± 14.89
Group (4) Sudden Death	26	29.30 ± 19.35	1.54 ± 1.26	8.05 ± 1.27	112.69 ± 11.26
p- value		0.706	0.940	0.964	0.013*

Value < 0 05 is significant

Table 6: Concentration of potassium / calcium according to postmortem time interval (n=100) according to Sturner's equation (PMI = 7.14 x K^+ conc. -39.1)

PMI	Potassium /calcium (mean ± SD)
Group 1 (0-24 hrs)	0.92 ± 4.27
Group 2 (24 -48 hrs)	1.22 ± 1.55
Group 3 (48 -72 hrs)	1.31 ± 0.50
Group 4 (72 – 96 hrs)	1.82 ± 0.21

due to contamination by bacteria, release of intracellular chemicals and postmortem metabolism. Among all the body fluids, vitreous humor has been found to be most suitable for the biochemical analysis to determine the postmortem interval. Vitreous humor is the only fluid which is unique, preferred because it is anatomically separated, resistant to putrefaction for a long time and most sterile. Even in most brutal homicide instances, caused by multiple stab injuries eyes are always spared.[15]

Application of various biochemical parameters as adjunct markers in the precise estimation of cause of death and time since death has been in practice for many years, accordingly levels of various parameters have been in use in the determination these aspects. Different chemical changes which may reflect in the variation of different biochemical parameters in fluids like blood, spinal fluid, and vitreous humor have been found to occur immediately and/or hours after death. Progressive changes in the concentration of these parameters may occur in fairly and orderly manner as the body disintegrates as the elapsing of time. Each parameter may undergo changes differently in a particular fluid as each parameter is different from other regarding its stability. Thus, determination of these changes could help the forensic experts/pathologists to ascertain time since death more precisely.[23]

Different biochemical parameters might not only help to diagnose the type of physiological or pathological condition prior to death but also the approximate duration the deceased person met his/her death. For the purpose of diagnosis of diabetes, parameters like glucose, acetone can give a clue to toxicologist/pathologist.[1] Many workers in the past have used the analysis of potassium to determine the postmortem interval.[13,24] To find out the condition like severe dehydration, levels of sodium and chloride would be of a great help[25] and also such as water intoxication.[26] So are the levels of urea and creatinine to diagnose renal

failure.[2]

Of course, the results of various biochemical parameters cannot be taken without exercising an element of caution as there are issues like reliability and reproducibility of various biochemical parameters, and many workers have reported problems with accuracy of various biochemical parameters in postmortem biological fluids. Many workers have come across problems such as variation in the results obtained from right and left eyes[13,27] and also due to the application of different analytical methods and instruments.[28]

In the present study vitreous humor was collected from the eyes of the dead bodies prior to the postmortem examination. Looking into the possibility of obtaining different results from the vitreous humor of different eyes when analyzed separately, as reported by many workers, in the present study we have pooled the vitreous humour of both the eyes and prepared a homogeneous fluid before taking for analysis.[13,27] Age and gender wise distribution from 15-77 years is given commest age group was 15-35, and 72 were males and 28 were females (Table 1). Distribution of subjects based on manner of death is given in Table 2. Levels of various biochemical parameters are given inTable 3. Concentration of biochemical parameters in the different age groups is given in Table 4. No significant difference in various biochemical parameters among different age groups in various biochemical parameters was observed. In females, glucose levels were significantly higher compared to males and chloride levels in males were significantly higher than that of females.

We have studied hourly changes in the vitreous calcium levels after death. Contradictory reports are available regarding the stability of calcium after death in the vitreous humor. Some researchers reported erratic results[29] and others found it to be stable.[30–32] Madea et al[33] showed the utility of vitreous calcium concentration in establishing postmortem interval. In the present study, we have observed a pattern of vitreous humor calcium in decreasing order from 0-90 hours of death.

As the results of vitreous glucose levels were erratic, the levels of glucose in blood along with acetone were considered. There was a decrease in the blood glucose concentration from 0-90 hours. Decrease in the blood glucose was significant (<0.05). Acetone was found in 04 subjects only. The presence of acetone in the samples gives an indication that the deceased person might had been suffering from diabetic ketoacidosis. We did not find any difference between various groups in the levels of urea, creatinine and chloride in vitreous humor.

Utility of Potassium levels to determine time of death: Determination of postmortem interval is very important, as sometimes this is the soul criteria to deal with this matter. In spite of the importance of the determination of postmortem interval, so far, no reliable parameter has been found regarding the determination of time since death. Cooling of body, postmortem staining, rigor mortis, decomposition changes, contents of stomach and bowls, contents of urinary bladder and circumstantial evidence were used to be in practice in olden days to find out the postmortem interval but all these give approximate time of death of wide range.

Analysis of potassium has been used many workers to determine the postmortem interval.[13,24] Looking into the importance of various biochemical parameters and necessity of establishing a reliable parameter to find out the post mortem interval, we have applied various equations to find out the utility of potassium and other parameters to determine this aspect. Various equations and formulae are used by a number of workers and one such is by Sturner equation (PMI (hours) =7.14 X K (meq/l)-39.1), developed in Western countries[34] and Yogiraj equation (PMI = 2.99 x K conc. - 6.26), developed in Indian conditions.[15]

Potassium is an intracellular cation and it's concentration in the eye is maintained by Na/Ka+ pump, potassium leaks out of cell leading to an increased postmortem level. Jaffee,[7] Hanson,[35] Leay and Farber,[36] Coe,[17] and Blumanfield[37] noted an increase in vitreous humor potassium level and on an average the rate of rise per hour was 0.17 meq /L. Similar findings were reported by various workers.[13,14,24,38–41]

Prasad et al[42] reported linear increase in the levels of vitreous humor potassium with the passing of time since death. In our results we have observed progressive increase of potassium from 0- 90 hrs. There was a linear rise of potassium values ranging from 7.04 meq/L to 15.81meq/, which is comparable to the values reported by Govekar.[43] In his study, the lower value of potassium was reported to be 3.56 meq/L, wherein in our study we have found higher levels of potassium i.e., 7.04 meq/L.

Yogiraj[15] reported that the levels of sodium and chloride remain constant for prolonged postmortem interval but there was a decrease of this value at the rate of 1mmol/ liter per hour at the later stage. Balasooriya et al[27] analyzed vitreous humor sodium, potassium and urate and the levels of these parameters showed higher significance and observed that the changes were according to the proportion to postmortem interval. There was a gradual increase in potassium and decrease in sodium in first 8.5 hours. In our studies also, we have found decrease in the levels of sodium from 0-90 and these results are in correlation with earlier mentioned studies.

In present study, we have used both Sturner and Yogiraj equations. Using the Sturner's equation we have placed the potassium value in equation and calculated the postmortem interval. In this study, we categorized subjects into four groups based on lapse of hour after death. First group was 0-24 hours, second was 24-48 hours, third was 48-73 hours and fourth was 73-96 hours. We have found increasing levels of potassium from 0-90 hours. Like other workers we have also observed a linear regression between

potassium level and postmortem interval. The average rise of potassium in this groups was 0.12 mEq/L per hour which is in correlation with the other earlier workers. Using Yogiraj equation, we have calculated PMI using the vitreous potassium. For this equation we have categorized subjects into two time slots. First group was 0 -24 hours and second was 24–48 hours. From using this formula, we have also found increasing potassium value from 0-48 hours. Here we have also observed linear regression between potassium level and postmortem interval like other workers. The average rise of potassium these groups was 0.42 meq/L per hour which is in correlation with other workers.

We have also studied the pattern of potassium/calcium, sodium/potassium. In case of sodium/potassium there was a decrease in the ratio, this is mainly due to the non-functioning of sodium-potassium pump, which leads to more and more leakage of potassium from intracellular compartment into the extracellular compartment leading to the increased levels of potassium. Correspondingly, we have observed a decrease in the levels of sodium over the period of death. Increase in vitreous potassium and decrease in sodium results into the decrease in the sodium/potassium ratio. We have also observed progressive increase in the ratio of potassium/calcium over a period of death.

7. Conclusion

In the present study subjects comprised of both males and females of age groups 15-25 (28), 26-35(28), 36-45 (16), 46-55 (16), 56-65(9) and 66-77 (3). We have used Sturner and Yogiraj equations to calculate change in electrolytes. We have observed the following:

1. There were 73% male and 27% females and commonest age group was 15 to 35 years.
2. No significant difference in these biochemical parameters was observed among these six groups but significant difference in glucose and chloride was observed between the genders.
3. There was a significant difference in various biochemical parameters with different manner of death. There was a possibility of some subjects had been suffering from diabetic ketoacidosis as the acetone was found to be positive in 4 out of 100 cases.
4. The levels of vitreous Potassium have been found to be significantly altered as the time progresses after death.
5. A progressive decrease in sodium/potassium and increase in potassium/calcium ratios was observed.

Laboratory Medicine has been playing crucial role in the diagnosis of various diseases in the antemortem scenario but the field of collaboration between Forensic Medicine and Toxicology and Laboratory Medicine has not been explored to its full potential to find out the time since death and cause of death.This pilot study gives enough indications of the importance of various biochemical parameters in the determination of cause of death and diagnosis of diseases.

Results obtained in this study clearly point towards the role of potassium with PMI but further studies are required to establish the utility of Potassium/Sodium and Potassium/Calcium ratio to find out the exact time since death.

8. Ethical Clearance

The present study was carried out after taking clearance from institutional ethical committee

9. Conflict of Interest

There is NO conflict of interest.

10. Authors' Contributions

Principal author is guide and assessing authority of the study. Corresponding author has helped in collection of samples and data entry. The third author is student and helped in collection and analysis of data. The fourth author is an ophthalmologist and guided how to collect the samples by aseptic precautions.

Acknowledgments

We are thankful to Dr Swati Patel, assistant professor, Community medicine for data analysis.

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Cite this article: Ramavataram DVSS, Sheikh MI, Pandey P, Sheikh K. Determination of postmortem interval and cause of death: Do the levels of biochemical parameters in vitreous humor provide an additional assistance to medico legal expert?. *Indian J Forensic Community Med* 2023;10(1):38-45.