ROLE OF VITREOUS HUMOR BIOCHEMISTRY IN FORENSIC PATHOLOGY

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ABSTRACT

Vitreous humor is a fluid that is relatively well protected from postmortem degradation and contamination. Due to its postmortem stability, vitreous humor has high utility in forensic pathology. Vitreous humor biochemical constituents, especially potassium, have been widely used in the postmortem interval (PMI) estimations. The time dependant rise of vitreous potassium levels in the postmortem period has been considered to be helpful in PMI determinations. The relative stability of vitreous biochemistry is useful in assessing the antemortem metabolic status and in predicting the antemortem serum biochemistry of an individual. However, the validity of vitreous biochemistry in forensic applications has been questioned in light of the reported concentration differences of various biochemical constituents in the same pair of eyes at identical PMI. This study hypothesized that the concentration of vitreous biochemical constituents in the same pair of eyes change at the same rate and this change that occurs in a time dependent fashion may be utilized in accurately estimating the PMI. It was further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry and may be a useful aid in establishing a postmortem diagnoses of hyperglycemia.

To test these hypotheses, vitreous humor samples were collected from a total of 103 autopsies (Female, 35 and Male, 68; Mean Age ± SD, 60.6 ± 17.6) conducted at Royal University Hospital morgue between January 2003 to February 2005. In 61 of these subjects, the precise time of death was known. Right and left eye vitreous humor samples were collected separately through a scleral puncture at the lateral

canthus. Most of the biochemical analyses were carried out immediately post-extraction. After centrifugation, the supernatant of the fluid were analyzed for sodium, potassium, chloride, calcium, magnesium, urea, creatinine, glucose and lactate on an LX-20 Analyzer (Beckman-Coulter). Osmolality was measured on an Osmometer model 3900 (Advanced Instruments Inc.). Vitreous humor hypoxanthine and xanthine were analyzed using a colorimetric method (Amplex® Red Xanthine/ Xanthine Oxidase Assay Kit, Molecular Probes Inc.). Vitreous humor lipid hydroperoxides were measured using the Ferrous Oxidation in Xylenol Orange assay version 2 (FOX 2). The data was statistically analyzed by paired t-test, linear regression analysis and Mann-Whitney test using Statistical Package for Social Sciences (SPSS) for Windows™ version 13.0.

The results of this study indicated that there were no significant between-eye differences for all of the vitreous biochemical constituents that were studied. It was observed that there was a significant correlation between vitreous potassium (R, 0.731; *P*, <0.0001), hypoxanthine, (R, 0.450; *P*, <0.0001), xanthine (R, 0.590; *P*, <0.0001), lactate (R, 0.508; *P*, <0.0001), calcium (R, 0.33; P, <0.01) and PMI. The corresponding formulae derived from the linear regression equations to estimate PMI were for potassium (6.41 (K^+) – 46.25), hypoxanthine (0.32 (Hypoxanthine) – 60.94), xanthine (0.14 (Xanthine) – 50.08), lactate (5.21 (Lactate) – 27.69) and calcium (200 (Ca^{2+}) – 380.4). On a comparison of the actual PMI and the estimated PMI calculated using the formulae derived from the linear regression correlationship, it was found that the lowest standard deviation and the highest correlationship was obtained for vitreous potassium. The mean paired actual and

estimated PMI values were significantly correlated for potassium (P, <0.0001), hypoxanthine (P, <0.0001), xanthine (P, <0.0001), lactate (P, <0.0001) and calcium (P, <0.01). Only vitreous potassium along with lactate and xanthine were significantly correlated with PMI in the same linear regression model. It was found that there was a highly significant correlation between antemortem serum and postmortem vitreous urea (R, 0.967; P, < 0.0001) and antemortem serum and postmortem vitreous creatinine (R, 0.865; P, <0.0001) concentrations. There was a significant difference (P, <0.05) between the postmortem vitreous glucose levels in the diabetic subjects as compared to the non-diabetic subjects. Vitreous lactate and lipid hydroperoxide levels did not exhibit any significant differences in these two diagnostic subgroups.

The results of the present study suggest that the previously reported between eye differences for various vitreous biochemical constituents in the same pair of eyes are insignificant so far as forensic applications are concerned. Vitreous potassium is a useful biochemical marker for PMI estimations. Vitreous hypoxanthine, xanthine, lactate and calcium are all significantly correlated with PMI and if used in conjunction with vitreous potassium may possibly enhance PMI estimations by narrowing the error margin. The knowledge of vitreous urea and creatinine levels are a useful index in predicting the antemortem metabolic and renal status of the deceased subject.

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To my grandmother, Mrs. Zaibunissa Mulla,

for teaching me the virtues of knowledge and inspiring me

towards its exploration and continued learning.

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ABBREVIATIONS

PMI	Postmortem Interval
FOX 2	Ferrous Oxidation in Xylenol range assay version 2
SPSS	Statistical Package for Social Sciences
K^+	Potassium
Ca^{2+}	Calcium
3-MT	3-Methoxy tyramine
Hx	Hypoxanthine
AMP	Adenosine Monophosphate
ROOH	Lipid hydroperoxides
IDDM	Insulin Dependent Diabetes Mellitus
NIDDM	Non-insulin Dependent Diabetes Mellitus
TBARS	Thiobarbituric Acid Reactive Substances
CSF	Cerebrospinal Fluid
TPP	Triphenylphosphine
H_2O_2	Hydrogen Peroxide
4AAP	4-aminoantipyrine
DCHBS	3, 5-dichloro-2-hydroxybenzene sulfonate
DMSO	Dimethyl sulfoxide
HRP	Horseradish Peroxidase
XO	Xanthine Oxidase
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulfuric Acid

BHT	Butylated hydroxytoluene
SD	Standard Deviation
SEM	Standard Error of Mean

1.0 LITERATURE REVIEW

1.1 VITREOUS HUMOR

1.1.1 Vitreous Humor Structure

The vitreous humor is located between the lens and the retina filling the centre of the eye. The vitreous humor, with an approximate volume of 4 mL, constitutes nearly 80% of the globe, making it the largest structure within the eye. However, relatively little is known about this important structure than any other part of the eye. During the 18th and 19th centuries, the four different popular theories of vitreous structure were alveolar theory by Demours, lamellar theory by Zinn, radial sector theory by Hanover and fibrillar theory by Bowman (Hart, 1992).

The alveolar theory suggested that the vitreous humor consisted of multitude of membranes oriented in all possible direction, enclosing alveoli containing the fluid portion of the vitreous. The lamellar theory suggested that the vitreous is arranged in a concentric lamellar configuration similar to the layers of an onion. The radial sector theory described a multitude of sectors approximately radially oriented about the central antero – posterior cone that contains Cloquets canal. The fibrillar theory described vitreous humor as fine fibrils that form bundles with nuclear granules. Apart from these four classical theories, some recent observations that have been made about the vitreous structure are relevant. Eisner's (Eisner, 1973) dissections of human vitreous found in the membranous structures ran from the

region behind the lens in a circumferential pattern, parallel with the vitreous cortex to insert at the posterior pole. He described these membranelles as funnels, which are packed into one another and diverge anteriorly from the prepapillary vitreous. These tractus were named according to their location. The peripheral vitreous tract separates the vitreous cortex from the inner vitreous, the tractus medianus extending to the ligamentum medianum of the pars plana, the tractus coronaries extends to the ligamentum coronarium from the pars plana and the innermost tractus hyaloideus arises at the edge of the lens and runs posteriorly along Cloquet's canal. Worst (1977) studied the preparations of dissected human vitreous and described the tracts of Eisner as constituting the walls of cisterns within the vitreous and termed these cisterns as hyaloid.

1.1.2 Vitreous Humor Biochemistry

The vitreous body is an extra cellular matrix that contains fibrillar structural proteins associated with varying amounts of hyaluronic acid and various types of proteins, glycoproteins and proteoglycans (Swann, 1980). Collagen is the major structural protein of the vitreous. Ayad and Weiss (1984) studied bovine vitreous collagen and their findings demonstrated that type II is the major vitreous collagen. Using contrast microscopy, Bembridge et al. (1952) had previously demonstrated the existence of fibrous filaments in the vitreous humor of several species including humans. Schwarz (1976) described the ultrastructure of the vitreous as a random network of thin uniform filaments, which were confirmed to be of collagenous nature by electron microscopic and X-ray diffraction studies. The organization of vitreous collagen fibrils within the vitreous and in relation to other molecular

components of the vitreous was described in detail by Schwarz (Schwarz, 1976). A large study of normal human eyes obtained at autopsy suggested that the collagen concentration in the vitreous gel in 70 to 90 years of age group was greater than in the 15 to 20 years age group. The finding was accounted due to a decrease in the vitreous gel volume that occurs with aging and a consequential increase in the concentration of the collagen fibers remaining in the vitreous gel (Balazs and Denlinger, 1982).

Hyaluronic acid is the major glycosaminoglycan present in the vitreous humor. In humans, hyaluronic acid first appears after birth and is synthesized primarily by hyalocytes. Comper and Laurent (1978) isolated hyaluronic acid and characterized this molecule and its configuration within the vitreous. An increase in the hyaluronic acid content of liquid vitreous and a corresponding decrease in the hyaluronic acid content of the vitreous gel due to increases in the amount of liquid vitreous and decreases in the amount of vitreous gel with advancing age has been suggested (Sebag and Balazs, 1989). Free amino acids are present in the vitreous at much smaller concentrations than that found in the plasma. Glycoproteins, the macromolecules of the ground substance of the vitreous, are mostly proteinaceous and contain only a minor carbohydrate component.

1.2 INTRAOCULAR FLUID DYNAMICS AND CONCENTRATION GRADIENTS

Vitreous humor chamber is one of the three large extra cellular compartments of a mammalian eye, the aqueous humor containing anterior and posterior chamber form the other two. The physiology and solute exchange mechanisms of intraocular fluids

and blood, within intraocular fluid compartments and surrounding intraocular tissues have been reviewed in detail by Bito (1977). During life, diffusion of low molecular weight solutes like glucose is unimpeded (Pirie, 1969). As a result, it is expected that under steady state conditions, measurable concentration gradients exist within the vitreous body, which will reflect the sites of entry and loss of solutes with the lens playing a key role in maintaining the normal gradients within the intraocular fluids (Bito, 1977).

1.2.1 Vitreous Humor Potassium

Vitreous potassium is actively transported into the posterior vitreous chamber from the lens (Bito and Davson, 1964). In the absence of the lens, low vitreous potassium levels were observed in animal eyes (Bito, 1977) and it has been suggested that potassium is actively transported out of the vitreous across the blood- retinal barrier (Bito and Davson, 1964). Hence, the potassium concentration in the vitreous adjacent to retina is lower and comparable to the levels in the cerebrospinal fluid and the brain (Bito, 1977). Under normal conditions, the net potassium gained from the lens by the vitreous chamber should be balanced by an equal loss from the vitreous to circulating blood through the retina (Bito and Salvador, 1970).

1.2.2 Vitreous Humor Magnesium

The posterior vitreous of canine, feline and rabbit had the highest magnesium concentration (Bito, 1977). A magnesium concentration gradient within the vitreous and the influx of magnesium across the blood retinal barrier occurring through an active transport process has been reported (Bito, 1970). As a result, it is expected

that the magnesium concentration levels are highest in the posterior vitreous chamber adjacent to the retina as compared to the anterior vitreous chamber.

1.3 UTILITY OF VITREOUS HUMOR IN ESTIMATING POST MORTEM INTERVAL (PMI)

1.3.1 Vitreous Humor Potassium

The body maintains a high concentration of potassium in the intracellular fluid. It is reported that the intracellular concentration of potassium is as high as 2 to 40 times the concentration of potassium within the plasma (Haas and Forbush, 1998). This high intracellular concentration is maintained by a balance between the electrical charges inside and outside the cell membrane and the active metabolic forces that pump the electrolytes selectively across the membrane. A return to equilibrium occurs after death at a steady rate because the pumping mechanism is inactive and the cell wall becomes a semi-permeable membrane that allows the potassium to leak through the membrane to approach equilibrium. The membrane leak occurs at a steady rate because of the mechanical limits of the membrane. The steady rate of potassium leak in the postmortem period provides a form of built in clock that allows a means of projecting back to the time of death and estimate the postmortem interval (PMI).

The postmortem increase in vitreous potassium concentrations has been widely studied and reported in literature with respect to it's correlation with the time since death or PMI. Naumann (1959) was one of the first workers to report a correlation and suggested that the sharp increase of potassium was mainly due to the autolytic permeability changes of the vascular choroid and retinal cell membranes.

Jaffe (1962) subsequently confirmed the postmortem rise of vitreous potassium and was the first to propose a statistical model for this relationship. Jaffe, using 36 determinations from 31 autopsies suggested a logarithmic correlation of PMI with time. Jaffe in his study also eliminated evaporation as a cause of postmortem rise of vitreous potassium by demonstrating the stability of sodium and chloride concentrations. Sturner (1963) based on a study sample of 91 autopsies proposed a formula of the straight line of best fit, calculated by the method of least squares as:

$$\textit{Time after death (in hours)} = 7.14\,[K^{+}] - 39.1$$

The standard error of this line of best fit was 4.7 hours with a correlation coefficient of 0.99. Adelson et al. (1963) demonstrated that the rise of potassium levels post mortem were linear with time. Sturner and Gantner (1964a) used this linear rise of potassium to establish the PMI. They suggested the first linear regression models for the relationship between vitreous potassium and PMI. Sturner and Gantner suggested that the confidence interval of their regression model was ± 4.7 hours (1 SD) and that there was no influence of an increasing PMI on this confidence interval. The period after the initial 24 hours since death was suggested to be the most useful working time of this test (Sturner and Gantner, 1964a). Another study comprising of 203 autopsies with a PMI of 3 to 310 hours reported wide dispersion in the vitreous potassium values after death (Hansson et al. 1966). After 120 hours of PMI, the potassium rise appeared to be constant suggesting a probable state of equilibrium had been reached by that time. The 95% confidence intervals obtained from the study were very wide and the authors suggested that the method of estimating PMI was far from ideal and at best could give only an indication of the

approximate time since death. Lie (1967) confirmed the findings of Sturner and Gantner (Sturner and Gantner, 1964a) with great precision. Based on the earlier formula proposed by Sturner and Gantner, Lie found differences exceeding 1.0 mEq/L (1.0 mmol/L) between the measured potassium and the expected potassium levels in only 4 cases out of a total of 88 autopsies. A narrow confidence interval of ± 1.7 hours in PMI estimation was obtained from the data of Adjutantis and Coutselinis (1972) based on a single potassium measurement within 12 hours of death. Interestingly, these authors further narrowed the confidence interval to ± 1.1 hours by refining the vitreous sampling method from each eye with a measured time interval, using the observed slope of the rise of potassium and extrapolating back to a zero time potassium value of 3.4 mEq/ L (3.4 mmol/L). However, the results from this technique were found to be valid only for the first 12 hours of PMI. Adjutantis and Coutselinis (1972) suggested that the actual time of death in about two-thirds of the cases would be within the limits of ± 1.1 hours of the estimated PMI. The authors suggested another advantage of the double sampling method in that if the second potassium value lies outside the limits of the estimated slope of regression line for potassium, it would suggest that the second sampling took place more than 12 hours since death. However, some later studies using the technique of Adjutantis and Coutselinis did not yield promising results. A postmortem increase of vitreous potassium proportional to the PMI was also noted in animal studies using dogs (Crowell and Duncan, 1974; Schoning and Strafuss, 1980). Crowell and Duncan (1974) similar to Jaffe (Jaffe, 1962) related the postmortem potassium rise to the logarithm of time. Agrawal et al. (1983) evaluated vitreous humor from 99 samples

from 50 autopsies and found a near perfect coefficient of correlation (R = 0.985) between vitreous potassium concentration and PMI.

A large pilot study evaluated 1427 cases with a PMI range of up to 35 hours (Stephens and Richards, 1987). The study estimated 95% confidence limits of inverse prediction for each point of potassium concentration to an approximate ± 20 hours and the coefficient of determination (R^2) was estimated to be 0.374. The linear regression equation obtained for potassium concentration (y) versus time (x) is:

$$y = 0.248x + 6.342$$

However, the authors of the study were skeptical of the practical relevance of the results beyond 35 hours PMI since the data of the study and its conclusion were limited to the range of 35 hours postmortem. The relationship between the postmortem rise of vitreous potassium and time since death was reviewed by Madea et al. (1989). They suggested that the differences in 95% confidence limits of various studies limited the usefulness of vitreous potassium as an aid in estimating the PMI. They reported sudden and hospital deaths after chronic diseases both yielded 95% limits of confidence in the range of ± 34 hours up to 120 hours postmortem. In cases with urea concentration less than 100 mg/dL (35.7 mmol/L), it was found that 95% confidence limits could be lowered to ± 22 hours. They reported 95% confidence limits of ± 20 hours after elimination of all cases with a terminal episode greater than 6 hours. In eliminating cases first of urea concentration >100mg/dl (> 35.7 mmol/L) and then duration of terminal episode greater than 6 hours, Madea et al. were able to narrow down the 95% confidence

limits from ± 34 hours to ± 22 hours and ± 20 hours respectively. Sparks et al. (1989) studied vitreous humor potassium from 91 persons for the purpose of accurate determination of PMI. Their data estimated 95% confidence limits of ± 10.5 hours in the first 24 hours of PMI and the confidence limits increased with a greater PMI. In the same study, the authors have devised a chemical method, which incorporates the vitreous potassium levels and 3-Methoxy tyramine (3-MT) levels in the putamen of the brain. Their study indicates that 3-MT method is as accurate if not more than vitreous potassium method in estimating PMI. They suggested that although 3-MT levels can be affected by specific cause of death and drugs taken antemortem, potassium and 3–MT levels together are the most accurate method of determining PMI (Sparks et al. 1989). A study by Zangg et al. (1997) suggested that vitreous potassium concentration changes over time could help estimate the PMI. They studied the vitreous humor and liver samples collected from hunter harvested elk (Cervus Elaphus) and mule deer (Odocillus Hemonus). Apart from potassium, calcium, chloride, sodium, urea nitrogen and selenium were determined and evaluated according to species, age, gender, geographic location and time elapsed since death. They concluded that vitreous humor analysis offered reliable biochemical information for up to 96 hours subsequent to death and reported a critical role for vitreous potassium in death time estimations.

1.3.1.1 Vitreous Potassium Based Formulae

Many equations and corresponding formulae have been reported in literature to precisely estimate the PMI based on post mortem vitreous potassium concentration. Many of these equations are based on the linear regression model on the assumption

that the postmortem increase in the vitreous potassium is fairly linear with time and changes at a constant rate.

The earliest and widely used equation was developed by Sturner (1963):

$$PMI\ (hours) = 7.14\ [K^+] - 39.1$$

The Sturner equation has been widely recommended for practical use (Coe, 1989). A primary disadvantage of this equation regarding a flat slope was reported in working with this equation (Madea et al., 1990a). Madea et al. (1989) devised their equation:

$$PMI\ (hours) = 5.26\ [K^+] - 30.9$$

The linear regression derived by Madea et al. had a steeper slope than that derived by Sturner (1963). Madea et al. suggested that Sturner's equation systematically overestimated the PMI because of the flat slope while their equation with a steeper slope had no systematic deviations. They concluded that when using vitreous potassium in estimation of the PMI, equations with a steeper slope should be preferred to avoid any systematic overestimation as a result of flatter slopes. Recently, James et al. (1997) devised an equation for estimation of PMI based on postmortem potassium concentrations:

$$PMI\ (hours) = 4.32\ [K^+] - 18.35$$

This equation yielded a mean overestimation of 0.4 hours with a SD (Standard Deviation) of 18 hours. On substituting their data in the equations of Sturner (1963) and Madea et al. (1990a), respective mean overestimations of 9 hours (SD of 30 hours) and underestimation of 2 hours (SD of 22 hours) were obtained.

Gamero et al. (1992) compared the equations developed by the various authors for PMI estimations based on vitreous humor potassium concentration. They were of the opinion that the equations formulated by Cespedes et al. (1979) underestimated the extrapolated times, while the equation proposed by Adjutantis and Coutselinis (1972) caused a systematic underestimation. They were not able to show any such discrepancy in the equations proposed by Madea et al. (1989) and Gamero et al. (1992). They suggested that these equations gave the most precise death time estimation with no systematic variations of PMI. Gamero et al., similar to Madea et al (1990a), suggested that the equation formulated by Sturner et al. (1963) tended to systematically overestimate the time since death.

Lange et al. (1994) analyzed original data from six different studies (Sturner et al. 1963; Hughes, 1965; Lie, 1967; Coe, 1969; Stegmaier, 1971; Madea et al. 1986). The objective of their study was to estimate PMI more precisely over the entire range of postmortem vitreous potassium and PMI studied than that obtained from any single original study. They used original data from the previous studies done through the period of 1963 to 1989 and comprising a total of 790 cases. They reported that the relationship between postmortem vitreous potassium and PMI was not completely linear and the residual variability of vitreous potassium as a function of PMI was not a constant. They developed a novel approach for modelling vitreous potassium and PMI to accommodate the non-linearities and changing residual variability. The original data from all six studies were combined to develop a single loess curve with 95% confidence bands. The estimated loess curve and confidence bands were used in an inverse prediction method to construct low, middle, high PMI

estimates at given values of vitreous potassium. Their study suggested that the reliability of estimated PMI decreases as vitreous potassium increased. Although the confidence bands surrounding the overall curve widened in the extreme high end due to there being fewer available data in that region, PMI estimates were far more precise over the entire range of vitreous potassium and PMI than those obtained from any single study alone.

1.3.1.2 Slope and Intercept of the Linear Regression

In spite of all the conflicting reports in literature regarding the different 95% confidence intervals and the best equation for practical use in PMI estimation, there is wide consensus on the linear increase of postmortem vitreous potassium with increasing PMI (Hansson et al. 1966; Jaffe, 1962; Bito, 1970; Adjutantis and Coutselinis, 1972; Komuro and Oshiro, 1977; Foerch et al, 1979; Blumenfeld et al. 1979; Forman and Butts et al. 1980; Farmer et al. 1985; Choo-Kang et al. 1983; Stephens and Richards, 1987). Some of these workers found the postmortem vitreous potassium rise to be biphasic in nature, the slope being relatively steeper in the initial hours after death (Coe, 1969; Choo-Kang et al. 1983).

Sturner (1963) and later Sturner and Gantner (1964a) based on their data of 54 coroner's cases reported a slope of 0.14 mmol/L per hour with an approximate zero hour intercept of 5.6 mmol/L. Adelson et al. (1963), reported a slope of 0.17 mmol/L per hour with an intercept of 5.36 mmol/L. Hughes (1965) suggested a biphasic linear pattern of postmortem vitreous potassium rise with an approximate slope of 0.12 mmol/L per hour with an intercept of 4.98 mmol/L. Another study that incorporated data from a larger range of PMI, some exceeding 250 hours, reported a

slope of 0.17 mmol/L per hour and a large intercept of 8 mmol/L (Hansson et al. 1966). Coe found the slope to be 0.332 mmol/L per hour in the initial 6 hours since death and there after the slope was found to be 0.1625 mmol/L per hour with an intercept at zero hours being 4.99 mmol/L and 6.19 mmol/L respectively. Adjutantis and Coutselinis (1972) who studied vitreous samples from a relatively small PMI range of 12 hours since death reported a rather large slope of 0.55 mmol/L per hour for the initial 12 hours of PMI. This finding is consistent with the earlier reports of a biphasic rise of postmortem vitreous potassium with a steeper slope in the initial hours as compared to the later PMI hours (Coe, 1969; Choo-Kang et al. 1983). Stephens and Richards (1987) with a large sample size of 1427 specimens taken from unrefrigerated bodies of all ages reported a linear increase of vitreous potassium with increasing PMI. The slope was 0.238 mmol/L per hour with a zero hour intercept of 6.342 mmol/L. Madea et al. (1989) also reported a linear relationship between vitreous potassium and PMI up to 120 hours. The slope was 0.19 mmol/L per hour with an intercept of 5.88 mmol/L. Recently, James et al. (1997) calculated a slope of 0.23 mmol/L per hour with an zero hour intercept of 4.2 mmol/L from their vitreous potassium data. A combined evaluation of the original data from six studies as done by Lange et al. (1994) and their subsequent use to fit a simple linear regression model, yielded a slope of 0.173 mmol/L per hour and an zero hour intercept of 5.695 mmol/L. This estimated slope was steeper than that originally reported by some authors (Sturner, 1963; Adelson et al. 1963) but flatter than that reported by Madea et al. (1989).

1.3.1.3 Factors Influencing Vitreous Potassium Concentration

The use of postmortem vitreous potassium concentration for the PMI estimation has been limited because of the different conclusions reached by different workers and the lack of uniformity in their equations. Certain factors can account for these marked variations in the behavior of postmortem vitreous potassium. These factors have been recently classified as external and internal (Coe, 1989). The external factors comprising of sampling, instrumentation and the ambient temperature of the body during the post mortem period. Some other factors like sampling and instrumentation differences may also account for the variations. The major internal factors possibly influencing the postmortem vitreous potassium are the age and the antemortem metabolic status of the individual. The effect of instrumentation and sampling on postmortem vitreous humor analysis has been reviewed in a later section, the influence of ambient temperature on postmortem vitreous potassium has been reviewed in this section.

Ambient Temperature: Many workers have documented the effect of temperature on post mortem vitreous potassium. Komuro and Oshiro (1977) were among the early investigators to suggest a significant influence of ambient temperature on vitreous potassium concentration. They found that the bodies present at warmer temperatures had increased rates of vitreous potassium than those present at lower temperatures. Similar observations have also been made by Coe (1973) who reported that elevated environmental temperatures accelerated the rise in potassium levels above that observed at room temperatures. The influence of temperature on postmortem vitreous potassium concentration has also been reported by Schoning

and Strafuss (1980). Based on their observations made on 60 adult mongrel dogs, the authors suggested that the potassium levels rose consistently with increasing temperatures even if the time was held constant. Mc Laughlin and Mc Laughlin (1987; 1988) studied the equine, cattle and swine vitreous humor to evaluate the influence of temperature on postmortem vitreous biochemistry. The effects of postmortem temperature were determined by incubation of samples at 4º to 37º C for varying time intervals. They reported that the vitreous potassium concentrations were generally more stable at lower temperatures and increased with time and temperature of the incubation of samples. However, other workers like Jaffe (1962), Adelson et al. (1963), Sturner (1963), Sturner and Gantner (1964a) did not observe any significant effect of temperature on postmortem vitreous potassium levels. The uniform rise of post mortem vitreous potassium slopes has also been reported across all temperature ranges (Bray, 1984). Although, postmortem vitreous potassium concentrations in the study were found to be lower in the cold weather groups than in other temperature ranges, these differences were not considered to be statistically significant.

Antemortem Metabolic Status: Madea et al. (1989) based on cases of sudden and chronic lingering deaths after chronic diseases suggested 95% limits of confidence in the range of ± 34 hours up to 120 hours post mortem. Since, urea was earlier shown to be the most stable of the vitreous constituents tested postmortem (Coe, 1969), it is considered to be an accurate indicator of antemortem urea retention. Therefore, any high urea values indicated ante mortem retention of urea rather than post mortem production. Madea et al. found an increase in regression slope

depending on the amount of urea nitrogen retention in the corpse. In cases with urea concentration less than 100 mg/dL (35.7 mmol/L), it was found that 95% confidence limits could be lowered to ± 22 hours. Through elimination of cases with the duration of terminal episode greater than 6 hours, they were further able to reduce the 95% confidence limits to ± 20 hours. By eliminating cases with urea concentration >100mg/dl and duration of terminal episode greater than 6 hours, Madea et al. were able to narrow down the 95% confidence limits from ± 34 hours to ± 22 hours and ± 20 hours respectively. Similarly, Sturner (1963) and Adelson et al. (1963) had suggested that the vitreous potassium values obtained from subjects with chronic disease conditions were more erratic than that obtained in individuals dying of acute conditions or trauma. This discrepancy may be a result of antemortem electrolyte imbalances caused by the chronic disease process and may explain the improved 95% confidence limits when Madea et al. (1989) eliminated cases with urea level > 100 mg/dL in their study. Since urea plays a central role in electrolyte metabolism, an impaired urea metabolism may be evident in chronic disease conditions. However, Hansson et al. (1966) found that the potassium content in the vitreous humor was unaffected by the underlying causes of death. This was quite in variance with the finding of later investigations (Madea et al. 1989).

AGE: Gardiner et al. (1990) reported an interesting observation from their study and suggested a significant influence of age on postmortem vitreous biochemistry. On evaluation of vitreous samples from chickens (*Gallus Domesticus*), the authors found that concentrations of inosine, hypoxanthine, uric acid, uracil and uridine changed significantly with increasing age. The study reported that as a general rule,

after the first week of life, the concentrations of these analytes decreased with increasing age and the increase in concentration with postmortem time reduced. The authors suggested that these changes resulted from inherent changes in metabolic rates with age. Crowell and Duncan (1974) studied vitreous humor of dogs and observed greater variation of potassium in the younger age group than the older dogs. The authors explained the variation might be due to the smaller globe of younger dogs, which posed difficulties in vitreous aspiration and may have led to contamination of the sample with aqueous fluid or blood. Blumenfeld et al. (1979) and Mason et al. (1980) also suggested that age influenced postmortem vitreous potassium concentrations, the regression slope being steeper for children than for adults. This may be attributed to the smaller globe diameter of the infants than the adults. It is important to consider the diameter of globe when making PMI estimations. Madea et al. (1994) explained that the concentration gradients that exist in the human eye aided the diffusion of various biochemical constituents from the retina to the vitreous humor in the postmortem period. The diameter of the globe, which represents the diffusion distance, is smaller in children than the adults and consequently the postmortem constituents are systematically higher in children because of a smaller globe diameter as per the Ficks law of diffusion. It is for this reason that the formulae used in PMI prediction for adults may not be suitable for use in children since there is a systematic overestimation of PMI owing to larger concentrations. The diameter and the length of the vitreous have been previously studied in different age groups and have been reviewed in great detail (Larsen, 1971).

OTHER INFLUENCING FACTORS: Crowell and Duncan (1974) based on their study on dogs had suggested that the weight of the dog that indirectly reflected the age of the dog may influence potassium concentrations in the postmortem vitreous humor. Madea et al. (1989) suggested possible effects of alcoholism at the moment of death on vitreous electrolytes. The authors suggested that alcohol might play a role in influencing postmortem osmolality of the vitreous fluid and therefore influence vitreous electrolyte concentrations.

1.3.2 Utility of Other Vitreous Constituents in PMI Estimation

Though potassium has remained the widely studied and reported vitreous constituent for PMI determinations, other constituents particularly hypoxanthine (Hx), calcium and magnesium have also been recognized for their utility in PMI estimations.

1.3.2.1 Vitreous Humor Hypoxanthine

The oxypurines hypoxanthine, xanthine and uric acid are the terminal stages of purine catabolism in man. Hypoxanthine, a biochemical marker of hypoxia that accumulates in body fluids during hypoxic degradation of Adenosine Monophosphate (AMP) within minutes after a hypoxic event (Saugstad and Gluck, 1982), is reported to increase in the postmortem period (Rognum et al. 1991; Madea et al. 1994).

Rognum et al (1991) emphasized the importance of hypoxanthine as an important vitreous humor constituent in estimating the PMI. They also emphasized the importance of temperature and its effect on increasing hypoxanthine concentrations in the postmortem vitreous humor. The study group involved

vitreous humor samples from 87 human subjects. Repeated sampling was performed in both the eyes and the bodies were kept at temperature ranging from 5°C to 23°C. There was a linear increase with time for both hypoxanthine and potassium. The slope for both hypoxanthine and potassium became steeper with increasing temperatures and there was a significant correlation (R = 0.93, $P<0.001$) between potassium and hypoxanthine levels in the whole group studied. In 19 of the subjects in whom the samples were obtained within 1.5 hours after death, the measured vitreous potassium levels were greater than the corresponding hypoxanthine levels. In the temperature group, the hypoxanthine levels were increased much more than the corresponding potassium levels. Rognum et al. suggested that since the scatter of the potassium levels was greater than that for hypoxanthine, the hypoxanthine levels were a better indicator for evaluating time since death, particularly in the initial 24 hours after death in subjects dying of non-hypoxic deaths. Rognum and coworkers obtained slopes of 4.2, 5.1, 6.2 and 8.8 μmol/L per hour for subjects stored at 5^o, 10^o, 15^o and 23^o C respectively. The authors tested the method in a case of triple homicide where the accused had confessed to the crime and had revealed the exact time of the crime. After applying this method to the known time of incident and comparing estimated time using potassium concentration, hypoxanthine concentration and brain temperature, the authors concluded that hypoxanthine levels better indicated the PMI than the other two. Madea et al. (1994) investigated if vitreous hypoxanthine or potassium established a better guide to detect time since death. Their investigations on 92 subjects with known PMI suggested that the correlation between vitreous potassium and PMI (R = 0.925) was much higher than

vitreous hypoxanthine and PMI (R = 0.714). They confirmed the linear rise of vitreous hypoxanthine beginning in the immediate postmortem period as was reported earlier (Rognum et al. 1991). Madea et al. argued that the previously reported higher correlation of vitreous hypoxanthine and PMI (Rognum et al. 1991) might have resulted from disturbed vitreous concentration gradients and intra ocular fluid dynamics due to repeated sampling technique employed in the study.

James et al. (1997) devised an equation for estimation of PMI based on post mortem vitreous potassium and hypoxanthine concentrations. The authors reported a slope of 0.23 mmol/L per hour and 3.2 μmol/L per hour for vitreous potassium and hypoxanthine respectively. They suggested that by combining the two variables (vitreous potassium and hypoxanthine), the precision of PMI estimations could be improved based on potassium alone or hypoxanthine alone or in combination of both. They proposed the following equations for potassium and hypoxanthine respectively:

$$PMI\ (\ hours\ ) = 4.32\ [K^{+}] - 18.35$$

$$PMI\ (\ hours\ ) = 0.31\ [Hx] + 0.05$$

They suggested that if the estimate of PMI is taken as the mean of the two estimates provided by their equations, the mean error is an over estimation of only 2 hours and a standard deviation (SD) of 15 hours.

A recent study reported that the cause of death could be an extra factor that modifies the relationship between postmortem vitreous potassium or hypoxanthine levels and gives greater precision in estimation of PMI (Munoz et al. 2002). The authors studied samples from 176 subjects, 35 subjects of hanging deaths and 141

subjects of non-hanging deaths. Accordingly, they proposed three different equations and formulae for all cases, hanging deaths and non-hanging deaths with R^2 (determination coefficient) values of 0.688, 0.818 and 0.663 respectively. The proposed formulae for casework for the three groups were formulated by the authors as follows:

$$PMI = 0.172\ [Hx] + 0.170 \quad \text{(All Cases)}$$

$$PMI = 0.153\ [Hx] - 0.368 \quad \text{(Hanging deaths)}$$

$$PMI = 0.178\ [Hx] + 0.278 \quad \text{(Non-hanging deaths)}$$

The authors reported improved precision in PMI estimation of hanging deaths with use of the formula specifically obtained from hanging deaths as compared to the estimations obtained from the general formula.

1.3.2.2 Vitreous Humor Calcium and Magnesium

The literature on the subject presents with conflicting reports regarding the utility of postmortem vitreous humor calcium and magnesium in making PMI estimations. Gregora et al. (1978) using the atomic absorption spectrophotometry estimated the proportion of potassium and calcium in the vitreous humor of 47 subjects. They observed a linear increase of both potassium and calcium with PMI. They suggested that the concurrent estimation of both potassium and calcium may help improve precision in PMI estimation as compared to the estimation from a single constituent alone. Gregora and coworkers cautioned against the use of calcium concentration in PMI estimations of deaths from metabolic disorders, brain injuries and strangulations where the utility of potassium alone was emphasized. Nowak and Balabonava (1989) suggested the concentrations of postmortem vitreous magnesium

and total calcium exhibited significant correlation with PMI only in deaths due to heart disease and asphyxia. However they could not correlate vitreous magnesium and calcium concentrations in other groups of death. The authors suggested that coronary occlusion can be considered to be a form of asphyxia and hypothesized that asphyxial deaths were followed by disturbances in the blood eye barrier that resulted in the increased inflow of calcium and magnesium into the vitreous humor. Further, Balabonava and Gras (1992) investigated postmortem concentrations of phenobarbital, magnesium and total calcium in vitreous humor obtained from sheep administered phenobarbital. The vitreous humor was obtained immediately from one of the eyes and after 3 days storage of head at 22º C from the other eye. A significant increase of phenobarbital and magnesium concentrations was observed in the postmortem period. They suggested that magnesium concentrations in postmortem vitreous humor could be useful in PMI evaluations in deaths with phenobarbital intoxication.

Some studies however betray this optimism in using vitreous magnesium as a predictor of PMI. Gregora et al. (1979) using the technique of atomic absorption spectrophotometry for estimation of sodium and magnesium in 50 deceased individuals did not yield any helpful conclusions regarding their utility in PMI estimations. The study did not find any dependence of postmortem concentrations of vitreous magnesium on PMI. Farmer et al. (1985) studied the levels of magnesium, potassium, sodium and calcium in post mortem vitreous humor from victims of fire fatalities and drowning in addition to human controls. They concluded that vitreous humor magnesium levels are not of any help in determining PMI. The study

suggested an average increase of only 0.036mg/L in magnesium concentration per hour, which is at the lower end of the spectrum of published ranges of 0.03-0.09mg/L per hour (Swift et al. 1974) and 0.1-0.22 mg/L per hour (Wheeler et al. 1983). The values obtained for calcium and sodium in the study data made this electrolyte metals practically useless to aid PMI estimations. 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. Wheeler et al. (1983) compared vitreous humor magnesium in alcoholics with that of non-alcoholics. They reported that no significant correlation existed between the postmortem vitreous magnesium levels and PMI in both the groups. Also, the levels of postmortem vitreous magnesium did not differ significantly in both the groups.

Similarly for calcium, Coe (1969) and Blumenfeld et al. (1979) have reported that there is no significant change in vitreous calcium concentration with increasing PMI. A near horizontal slope of the regression line was reported for postmortem calcium with no significant change with increasing postmortem interval (Dufour, 1982). Madea et al. (1990b) disagreed on the utility of vitreous calcium concentration in establishing PMI. They refuted an earlier study (Nowak and Balabonova, 1989) that had suggested a significant relationship between vitreous calcium concentration and PMI in asphyxial deaths. Madea et al. argued that though a significant correlationship ($R = 0.356$) existed between these two parameters, the range of scatter grossly limited its practical usefulness.

1.4 VITREOUS HUMOR BETWEEN-EYE DIFFERENCES

The various factors discussed may have a key role in the various disagreements regarding the utility of vitreous humor in PMI estimations. However, perhaps the most important concern in utilizing vitreous biochemistry for crucial forensic pathology determinations arises from the observed between eye differences in the same pair of eyes at identical PMI. Many researchers have assumed that the vitreous biochemical concentrations are identical and postmortem changes occur at the same rate in both the eyes. Recent observations have indicated that these presumptions may not be entirely true and between eye differences at the same PMI have been documented. If these differences were to exist, it would grossly undermine the value of vitreous biochemistry in various forensic pathology applications. Balasooriya et al. (1984) reported significant differences in various vitreous biochemical constituents from the same pair of eyes at identical PMI. The authors observed that each of the eyes exhibited independent values and nearly 19% of the results for vitreous potassium varied by more than 10% from the mean of the two values. Out of a total of 59 pairs of eyes, only six pairs had the same potassium concentration. Similar differences were also observed for vitreous sodium where 10% of the results varied by greater than 5% of the mean, vitreous urate where at least 19% of the cases had differences greater than 12% of the mean values of the two eyes. Subsequently, Madea et al. (1989) confirmed these findings and reported deviations up to 10% of the single values of both the eyes in the analysis of potassium, sodium, chloride and calcium. The authors, however, did not observe any such deviations for urea. Pounder et al. (1998) from a later study reported that between eye differences

in potassium varied from 0 to 2.34 mmol/L or 0% to 21.8% of the mean. The authors suggested these differences to be significant and erratic, thereby questioning the practical usefulness of vitreous humor in evaluation of PMI. On the contrary, the authors reported that the differences observed for sodium and chloride were tolerable using their methodology. Tagliaro et al. (2001) explored potassium concentration differences in the vitreous humor of two eyes using micro sampling technique with capillary electrophoresis. The authors reported that no significant differences existed in potassium concentration of the same pair of eyes at identical PMI, thus strengthening the application of vitreous humor as an important tool for PMI estimations.

1.5 ANTEMORTEM SERUM AND POSTMORTEM VITREOUS BIOCHEMISTRY CORRELATION

Antemortem serum biochemical alterations are a regular feature in many diseases and the availability of the antemortem serum levels are useful in establishing a postmortem diagnosis. However, antemortem serum biochemistry may not be available in many cases. In such instances, the presence of an isolated biochemical fluid that is free from postmortem changes or contamination may be very useful. Vitreous humor is one such body fluid and its biochemical profile closely mimics the blood chemistry (Coe, 1969).

The biochemical concentrations of blood obtained almost immediately after death and postmortem vitreous urea nitrogen, sodium, potassium and calcium were evaluated at different PMI in various bovine breeds (Wilkie and Bellamy, 1982). Near identical levels were obtained for sodium and urea in both the fluids of the

bovine. The vitreous calcium levels were 60% of the antemortem serum levels and the serum potassium levels were consistently higher than the normal vitreous levels obtained in the immediate postmortem period. A gradual increase of vitreous potassium was noted at various PMI and reached 50% of the immediate post mortem concentrations at 24 hours PMI. The same study, based on observations on dogs, also reported that several hours were required for equilibration of elevated blood urea levels with vitreous humor urea. The slow postmortem changes of vitreous humor calcium have been previously identified (Coe, 1969; Blumenfeld et al. 1979). The reported postmortem stability of vitreous humor calcium contradicts findings of variability up to 2 mg/dL (0.5 mmol/L) in the same pair of eyes with vitreous humor extracted 24 hours apart (Swift et al. 1974). Also, a linear increase of postmortem vitreous calcium that was utilized in estimating PMI in certain classes of death has been reported (Gregora et al. 1978; Nowak and Balabonova, 1989). Dufour (1982) observed that vitreous humor calcium did not change significantly with increasing PMI and the slope of the regression line was almost horizontal at 0.013 mg/dL per hour (0.0033 mmol/L). Dufour reported that the serum calcium was higher than vitreous calcium in almost all the cases with a difference range of 0.8 to 3.8 mg/dL (0.2 to 0.95 mmol/L). Though the vitreous calcium concentration did not change significantly postmortem, the vitreous and serum calcium correlation was poor and not significant. Therefore, it was suggested that vitreous calcium estimation was not useful in evaluating ante-mortem serum calcium disturbances.

The vitreous chloride levels were found to be constant in the vitreous humor up to 70 hours PMI (Gregora, 1985). Based on the observation of postmortem stability of vitreous chloride concentrations, it was suggested that post mortem vitreous humor chloride levels could help derive conclusions about the antemortem chloride status of an individual. Gregora (1984) reviewed the creatinine and urea in vitreous humor post mortem and reported that the ante-mortem serum urea and creatinine levels corresponded well with the values obtained in the postmortem vitreous humor.

Lincoln and Lane (1985) studied postmortem vitreous humor magnesium concentration in 97 healthy cattle and concluded that the postmortem vitreous humor magnesium concentration helps in diagnosing magnesium imbalances after death for a period of at least 48 hours if the environment temperature did not exceed 23° C after 24 hours. The study suggested that at low environmental temperatures (4^o C) the magnesium concentration was relatively stable as opposed to significant reductions in the postmortem vitreous concentrations at high temperatures (30^o C). In a comparison of fresh equine serum and vitreous humor chemistry obtained immediately after slaughter, urea nitrogen, sodium and potassium concentrations were highly similar (McLaughlin and McLaughlin, 1988). The ratio of vitreous to serum concentration was 0.91, 0.96 and 0.83 for urea, sodium and potassium respectively. The vitreous urea values were reported to be stable up to 48 hours at 20^o C. However, after 24 hours of incubation at 37^o C the values were found to be higher which increased further with a prolonged incubation. Similarly, stable concentrations of sodium were reported up to 48 hours at low temperatures but

stability lasted for only 12 hours when samples were incubated at 37^{o} C. The authors also reported a remarkable stability of post mortem vitreous calcium and chloride levels for intervals up to 48 hours at 20^{o} C. A strong linear correlationship was derived for antemortem serum values and postmortem vitreous urea and creatinine concentrations in animal species including bovine urea (R^2 = 72.2), creatinine (R^2 = 61.1) and canine urea (R^2 = 99.8), creatinine (R^2 = 92.3) at 24 hours PMI (Hanna et al. 1990).

1.6 UTILITY OF VITREOUS HUMOR IN EVALUATING CAUSE OF DEATH

Establishing the cause of death is one of the frustrating challenges faced by a forensic pathologist, particularly in situations with limited antemortem information about the deceased individual. The postmortem analysis of the vitreous fluid has been suggested to exhibit characteristic findings in certain classes of death and in certain cases, post mortem vitreous humor analysis may be an important adjunct to confirm an ante mortem diagnosis.

1.6.1 Postmortem Vitreous Biochemistry in Diabetic Subjects

In clinical practice, biochemical markers such as serum glucose are used to diagnose diabetes mellitus. At autopsy, this marker is of limited value due to substantial and capricious fluctuations in blood glucose levels after death. The vitreous humor of the eye is an isolated fluid and is far less susceptible to postmortem chemical fluctuations as compared to blood. Coe (1969) indicated that the antemortem vitreous glucose concentrations were 85% of the plasma glucose values. This contradicted the earlier suggested 50% of plasma glucose concentration (Sturner and

Gantner, 1964b). Assuming the proposed value of Coe to reflect antemortem glucose concentrations, a vitreous glucose level above 9.44 mmol/L corresponding to blood glucose > 11.1 mmol/L indicates diabetes mellitus.

Many previous studies have addressed the postmortem concentrations of vitreous humor glucose (Sippel and Mottonen, 1982; Bray et al. 1983; Peclet et al. 1994). A rapid decrease in vitreous glucose levels is caused in the postmortem period due to the anaerobic degradation or glycolysis. The decrease may be up to 35% in the first hour to 70% after 6 hours PMI (Bray et al. 1983). The whole process of glycolysis is completed by 3.5 to 7 hours after death (Hamilton-Paterson and Johnson, 1940) and is time (Bray et al. 1983) and temperature dependent (Bray et al. 1984), with cold temperatures inhibiting glycolysis and delaying the completion of the glycolytic process. The decrease in glucose levels stops at approximately 18 hours PMI. The postmortem decrease in glucose levels makes prediction of ante-mortem glucose status extremely difficult. Schoning and Strafuss (1980) observed a rapid fall of postmortem vitreous glucose levels in dogs and emphasized the use of vitreous humor in diagnosing a hyperglycemic state but not the hypoglycemic state. In a study by Canfield and colleagues (2001), postmortem vitreous glucose levels greater than 5 standard deviations of the normal values were considered as abnormal. They identified 9 cases (5% of total 192 cases) as having abnormal glucose levels and all were previously identified in life as having high glucose levels. Moreover 8 of these 9 cases were previously diagnosed with a diabetic condition. Dimaio et al. (1977) reviewed cases of sudden and unexpected deaths after an acute onset of diabetes mellitus where postmortem vitreous glucose

analysis was critical towards a correct diagnosis. Khuu et al. (1999) described a case of undiagnosed diabetes, where the estimation of vitreous glucose of 502 mg/dL (27.9 mmol/L) was the key in identifying the diabetic condition. The authors stressed the importance of vitreous humor hemoglobin A_{1c} value as a definitive indicator of prolonged hyperglycemia. Irwin and Cohle (1988) illustrated the deaths of two obese women, unknown to be diabetic previously, dying of diabetic ketoacidosis. The authors suggested that the post mortem vitreous biochemistry was the key in diagnosing sudden deaths due to diabetes in the absence of previous history or significant disease of internal organs.

1.6.1.1 Vitreous Glucose and Lactate Measurements in Diabetic Subjects

The postmortem vitreous humor glucose levels are limited by their dependence on time and environmental temperature. It is therefore essential that additional glycemic markers be utilized to supplement the vitreous glucose levels in establishing a postmortem diagnosis of a hyperglycemic condition. In anaerobic conditions, such as is prevalent after cessation of life, glucose is converted to lactate. A diagnosis of fatal diabetes mellitus and hypoglycemia with cerebrospinal fluid (CSF) glucose and lactate values (Traub, 1969), and in alcoholics, estimation of glucose and lactate together helps in the analysis of diabetic deaths and metabolic disorders (Brinkmann et al., 1998). Earlier investigators had stressed on the usefulness of detecting glucose and lactate values in combination to help in the diagnosis of antemortem hyperglycemia (Sipple and Mottonen, 1982; Peclet et al., 1994). High vitreous glucose and lactate values, both individually and together were suggested to indicate antemortem hyperglycemia (Sipple and Mottonen, 1982).

The observed mean of combined vitreous glucose and lactate was 277 mg/dL (15.4 mmol/L). Interestingly, the PMI had no significant effect on the combined glucose and lactate values 2 to 8 days after death. Peclet et al. (1994) studied 328 autopsies and confirmed the findings of Sipple and Mottonen. They further established that vitreous glucose and lactic acid and blood levels of acetone were valuable indicators of ante-mortem hyperglycemia in diabetics. However, they observed a lower mean of combined values (149 mg/dL or 8.27 mmol/L) and attributed this to the different methods and larger sample size of their study. In another study, a mean of combined glucose and lactate values of 289.5 mg/dL (16.07 mmol/L) was observed in the diabetics as compared to 147.8 mg/dL (8.20 mmol/L) observed in the non-diabetics (Osuna et al. 2001). The highest levels of glucose and lactate were observed in the diabetic group with statistically significant differences between the two groups.

Osuna et al. (1999) evaluated the usefulness of determination of fructosamine in the vitreous humor for confirming the presence of antemortem hyperglycemia. In a study of 92 cadavers, they assigned cases to two groups based on antemortem diagnosis of diabetes mellitus. They observed statistically significant differences for glucose and fructosamine concentrations between the two groups with the highest values recorded in subjects with a previous diagnosis of diabetes mellitus. However, the sum values of vitreous glucose and lactate than fructosamine alone were found to be better predictors of antemortem diabetes (Osuna et al. 2001).

1.6.1.2 Lipid Hydroperoxides in Diabetic Subjects

Oxidative stress has been proposed to be an important component in diabetes mellitus and its complications (Baynes, 1991). Lipid hydroperoxides (ROOH), the

reaction products of oxidative damage are one of the parameters for lipid peroxidation measurements and are helpful in the assessment of oxidative damage in vivo (Gutteridge and Halliwell, 1990). Plasma lipid hydroperoxides were shown to be significantly increased (7.23 ± 2.11 vs 2.10 ± 0.71 μmol/L, $P < 0.001$) in Type 1 or insulin dependent diabetes mellitus (IDDM) subjects compared to controls (Santini et al. 1997). The level of plasma lipid hydroperoxides was independent of the metabolic control reflected by the HbA_{1C} and the triglyceride or cholesterol levels of the diabetic subjects. A similar increase in plasma lipid hydroperoxides (9.4 ± 3.3 vs 4.1 ± 2.2 μ mol/L, $P < 0.001$) was also observed in Type 2 or non-insulin dependent diabetes mellitus (NIDDM) patients when compared to controls (Nourooz-Zadeh et al. 1997) with no association of plasma lipid hydroperoxide levels with the diabetic complications or smoking profiles of diabetics. The authors suggested that since lipid hydroperoxides did not show any significant correlation with the diabetic complications, their increase possibly occurs early in disease and not as their consequence.

1.6.1.2.1 Measurement of Lipid Hydroperoxides

Two different indices of lipid peroxidation are conjugated dienes and lipid hydroperoxides that are generated at various stages of the peroxidation cascade (Santini et al. 1997). Although the biochemical importance of lipid peroxidation has been well known their measurement has been difficult (Halliwell, 1996). The traditional assays of conjugated dienes (Princen et al. 1992) or thiobarbituric acid reactive substances (TBARS) (Janero et al. 1990) are limited by confounding factors (Corongiu et al. 1994) and the lipid content of the sample respectively (Janero et al.

1990). The ferrous oxidation in xylenol orange version 2 (FOX 2) assay based upon the oxidation of ferrous ions to ferric form under acidic conditions measures the whole plasma lipid hydroperoxides (Nourooz-Zadeh et al. 1994). The ferric ions generated are complexed by the ferric ion indicator, xylenol orange that generates a blue-purple complex with an absorbance maximum at 550 to 560 nm. The signal is authenticated using triphenylphosphine (TPP) as a specific ROOH reductant. The mean normal plasma values were estimated to be approximately 2-4μmol/L (Nourooz-Zadeh et al. 1994, 1997; Santini et al. 1997) and up to three fold higher values in diabetics as compared to non-diabetic controls have been reported (Nourooz-Zadeh et al. 1994, 1997; Santini et al. 1997).

1.6.2 Diagnoses of Other Causes of Death

High vitreous humor sodium concentrations in subjects dying of a brain contusion and increased magnesium concentrations in subjects with chronic liver conditions have been reported (Gregora et al. 1979). A postmortem rise in the vitreous humor osmolality, independent of the postmortem solute status, has been suggested (Bray, 1984). The usefulness of postmortem vitreous biochemistry in deaths due to uremia, dehydration and hyponatraemia has been proposed (Devgun and Dunbar, 1986). The antemortem hydration and the renal status of a subject reflected by simultaneous elevation of vitreous humor sodium (> 155 mmol/L) and chloride (> 132 mmol/L) has been reported (Forman and Butts, 1980). The vitreous urea and creatinine concentration are helpful in the antemortem renal status assessment as are increased levels of calcium in diagnosis of ante-mortem hypercalcemia (Choo-Kang et al. 1983). Additionally, detection of bilirubin in the vitreous humor has been reported

to be always pathological, most likely indicating liver disease. Vitreous osmolality and osmolar gap estimation may be useful adjunct analysis to aid in the diagnosis of deaths in subjects with presence of osmotically active substances like alcohol or other substances (Devgun and Dunbar, 1986). Sudden death in alcoholics with no apparent cause of death determined at autopsy is a practical issue faced by many practicing pathologists. Magnesium deficiency though well known in alcoholics (Sullivan et al. 1969; Lim and Jacob, 1972) is not reflected in the postmortem vitreous humor and is not helpful in determining the cause of death in this group (Forman and Butts, 1980; Wheeler et al. 1983). In alcoholics with no apparent pathological abnormalities, decreased sodium and chloride levels were evident (Forman and Butts, 1980). Recently, low postmortem vitreous magnesium levels were identified as useful biochemical markers in the postmortem diagnosis of hypomagnesaemic tetany in adult sheep (McCoy et al. 2001). Magnesium concentrations of <0.60 mmol/L and <0.65 mmol/L were observed to indicate a diagnosis of hypomagnesaemic tetany in vitreous humor collected fresh and at 24 hours PMI respectively.

1.7 HYPOTHESIS

It is hypothesized that certain vitreous humor biochemical constituents in the postmortem period increase in a time dependant manner and this increase may be utilized to accurately estimate the PMI. It is also hypothesized that the concentration of vitreous biochemical constituents in the same pair of eyes changes at the same rate and fashion at identical PMI. It is further hypothesized that postmortem vitreous humor biochemistry closely mimics antemortem serum biochemistry of an

individual and may also be a useful aid in establishing a postmortem diagnoses of diabetes mellitus or hyperglycemia.

1.8 OBJECTIVES

The main objectives of the study were to investigate:

(i) The utility of vitreous biochemistry in estimating PMI

(ii) The important vitreous biochemical markers that can aid PMI estimation

(iii) The between eye differences of various vitreous humor biochemical constituents

(iv) The correlationship between antemortem serum and postmortem vitreous biochemistry

(v) The utility of antemortem hyperglycemic markers in postmortem diagnoses of diabetes mellitus

To achieve these objectives, the following studies were undertaken:

Vitreous humor was collected from the eyes of a total of 103 autopsy subjects. In 61 of these subjects, the precise time of their death was known. Vitreous humor sodium, potassium, chloride, magnesium, calcium, urea, creatinine, glucose, lactate, osmolality, hypoxanthine, xanthine and lipid hydroperoxide levels were measured in all of these subjects. The biochemical measurements were correlated with the pertinent medical history and autopsy diagnoses obtained from the subject's medical records. Linear regression correlation analysis was performed to derive appropriate formulae for all the constituents that were significantly correlated with PMI.

2.0 MATERIALS AND METHODS

2.1 SUBJECT CHARACTERISTICS

Vitreous humor samples were collected from a total of 103 pairs of eyes during January 2003 to February 2005 from the autopsies conducted at Royal University Hospital morgue. 68 subjects in all were males and 35 were females in the age group of 16 to 95 years (Mean ± SD, 60.6 ± 17.6). A smaller sub-group of 61 subjects, 39 male and 22 female subjects in the age group of 16 to 95 years (Mean ± SD, 62.5 ± 15.7), from the total of 103 subjects had a precisely documented time of death. The post mortem interval (PMI) range in the second group was 4.5 to 84.3 hours (Mean ± SD, 27.9 ± 16.5). The autopsy subjects in the smaller group mainly comprised of hospital deaths where the time of death was precisely known. A few subjects (n = 9) were non-hospital deaths with recorded time of death based on coroners report and witness accounts. The study population therefore comprised of medico legal and non-medico legal cases. The causes of death in the study group included complications arising from metabolic, cardiovascular, respiratory, malignancy and trauma causes.

In a few cases, the sample was insufficient for the biochemical analyses of all the vitreous constituents, therefore analyses for some analytes were not carried out. In certain other cases, due to some analytical problems like interference and the measurement for particular analytes being outside the linear accuracy range of the

instrument, precise and reliable results were not available. Samples in which the measurement for only one eye was available were omitted from the studies. In total, 100 paired measurements for sodium, 85 for potassium, 100 for chloride, 102 for calcium, 87 for magnesium, 98 for urea, 100 for creatinine, 85 for glucose, 77 for lactate, 78 for osmolality, 96 for hypoxanthine, 70 for xanthine and 79 for lipid hydroperoxides were possible. All the pertinent medical and health information including the autopsy findings and diagnoses were recorded from the subject's health records.

2.2 VITREOUS HUMOR COLLECTION

The vitreous humor samples were collected by the method proposed by Coe (1989). Briefly, using a 12 mL syringe and a needle, a scleral puncture was made on the lateral canthus and the total extractable vitreous humor was aspirated from each eye separately. Adequate care was taken to gently aspirate the fluid and avoid tearing of any loose tissue fragments surrounding the vitreous chamber. On an average, an approximate amount of 2.5 mL was collected from each subject. Only crystal clear liquid free from tissue contamination and fragments was used in the study.

2.3 BIOCHEMICAL ANALYSES

The samples after collection were immediately transferred in a red-top tube, labeled and transported to the clinical chemistry laboratory for analysis. Most of the biochemical analyses were carried out immediately post-extraction. The sample was analyzed for sodium, potassium, chloride, calcium, magnesium, urea, creatinine, glucose and lactic acid. These analyses were carried out on the same day as collection. Before analysis the vitreous samples were centrifuged at 2050 g for 10

minutes. The analysis for these constituents was carried out using a Beckman Coulter LX20 Automated Analyzer. In addition vitreous humor was also analyzed for hypoxanthine, xanthine, lipid hydroperoxides and osmolality. Vitreous hypoxanthine and xanthine were measured using a colorimetric method (Amplex® Red Xanthine/ Xanthine Oxidase Assay Kit, Molecular Probes, Inc., Eugene, OR, USA). Vitreous lipid hydroperoxides were measured using the Ferrous Oxidation in Xylenol assay version 2 originally described by Nourooz-Zadeh et al. (1994). Vitreous osmolality was measured using the freezing point method on Osmometer model 3900 (Advanced Instruments Inc.).

2.3.1 Biochemical Analyses- LX 20 Automated Analyzer

The principles of biochemical analyses in the LX-20 automated analyzer are as follows:

Sodium: The sodium electrode is made of lithium-sodium-aluminium-silicate glass. It is essential that the outer layer of the electrode be adequately hydrated. When the sample/ buffer mixture contacts the electrode, sodium ions in the sample undergo an ion exchange process with the same sodium ions in the hydrated layer of the electrode. This causes a change in the electrode potential. A reference reagent that contains sodium ions is introduced into the flow cell after the sample cycle. The same ion exchange process takes place. The differential potential (voltage) between sample and reference reagent is used to calculate the sodium concentration. **Potassium**: The potassium ion selective electrode is made of vanilomycin. The physical structure of the vanilomycin ionophore is such that its cavity is nearly equal to the diameter of the potassium ion. This allows for the irreversible binding

of the potassium to the vanilomycin. When the sample/ buffer mixture contacts the electrode, changes in electrode potential occur as the potassium ions react with the vanilomycin. The potential change is referenced to the sodium reference electrode. A reference reagent that contains potassium ions is introduced into the flow cell after the sample cycle. The same ion exchange process takes place. The differential potential (voltage) between sample and reference reagent is used to calculate the potassium concentration in the sample.

Chloride: The chloride electrode consists of a soluble silver chloride compound. When the sample/ buffer mixture contacts the electrode, chloride ions produce a shift in the silver-chloride reaction equilibrium. The shift results in a change in electrode potential. A reference reagent that contains chloride ions is introduced into the flow cell after the sample cycle. The same chemical equilibrium shift process takes place. The differential potential (voltage) between sample and reference reagent is used to calculate the chloride concentration in the sample.

Calcium: The ion selective calcium electrode measures unbound free calcium ion in the solution. Total calcium can only be calculated from free calcium when the molar ratio between free and total calcium is constant. This constant molar ratio is achieved by the buffered solution, which contains strong calcium complexing agents. The calcium electrode consists of a calcium ionophore membrane cast on a solid support. On contact of the sample/ buffer mixture with the electrode, changes in electrode potential occur as calcium ions react with the ionophore. A reference reagent that contains calcium ions is introduced into the flow cell after the sample

cycle. The differential potential (voltage) between sample and reference reagent is used to calculate the calcium concentration in the sample.

Magnesium: Magnesium combines with calmagnite to form a colored magnesium-calmagnite complex in a timed endpoint reaction. The absorbance at 520 nm is directly proportional to the magnesium concentration in the sample.

Urea: The urea in the sample is converted from the non-ionic form to the ionic form (ammonium ion and bicarbonate) by urease activity in the reagent. The conductivity of the ionic solution is measured over time. The rate of conductivity is proportional to the concentration of urea in the sample.

$$\text{Urea (non-ionic)} \xrightarrow[\text{urease}]{} \text{Ammonium ion + Bicarbonate}$$

Creatinine: Creatinine from the sample combines with the picric acid reagent to produce a red color complex. Absorbance readings are taken at 520 nm 19 to 25 seconds after the sample was added to the reaction cup. The change in absorbance is directly proportional to the creatinine concentration.

Glucose: Glucose oxidase in the reagent catalyzes glucose in the sample to yield peroxide and gluconic acid. This reaction consumes oxygen. The rate of oxygen consumption is directly proportional to the glucose concentration in the sample.

Lactate: Lactate oxidase converts lactate to pyruvate and hydrogen peroxide (H_2O_2) in a timed endpoint reaction. The hydrogen peroxide reacts with 4-aminoantipyrine (4AAP) and 3, 5-dichloro-2-hydroxybenzene sulfonate (DCHBS) in a reaction catalyzed by peroxidase to produce a chromophore.

$$\text{L-lactate} + O_2 \xrightarrow[\text{lactate oxidase}]{} \text{Pyruvate} + H_2O_2$$

$$H_2O_2 + \text{DCBSA} + 4\ \text{AAP} \longrightarrow \text{Chromophore}$$

The absorbance at 520 nm is directly proportional to the lactate concentration in the sample.

2.3.2 Measurement of Vitreous Humor Osmolality

Osmolality was measured using the freezing point osmometry principle using the Advanced Osmometers. Advanced osmometers are devices for the extremely precise determination of the concentration of solutions by means of freezing point measurement. The sample is supercooled within a cooling fluid. A rapid stir mechanism is used to initiate crystallization. The temperature will then rise due to latent heat of crystallization. A thermistor (temperature dependent resistor) reading is noted and comparison with standards allows calculation of osmolality. Advanced osmometers use high precision electronic thermometers to sense the temperature, control the degree of supercooling and freeze induction, and measure the freezing point of a sample.

2.3.3 Measurement of Hypoxanthine and Xanthine

The remaining vitreous humor sample after the analyses of all the above constituents was stored in 5 mL plastic eppy tubes frozen at - 70°C for measurement of hypoxanthine, xanthine and lipid hydroperoxides content.

Hypoxanthine and Xanthine were analyzed using a commercial kit Amplex® Red Xanthine/ Xanthine Oxidase Assay Kit (Molecular Probes, Inc., Eugene, OR, USA) that utilized the colorimetric method. The commercial kit provides a highly sensitive method for detecting hypoxanthine, xanthine and xanthine oxidase activity. In the assay, xanthine oxidase catalyzes the oxidation of purine bases, hypoxanthine or xanthine, to uric acid and superoxide. The

superoxide spontaneously degrades to hydrogen peroxide. The peroxide, in the presence of horseradish peroxidase, reacts stoichiometrically with the Amplex Red reagent to produce the product resorufin. Resorufin has absorbance and fluorescence emission maxima at 563 and 587 nm respectively. Because the extinction co-efficient of the product is very high (E=54000 $cm^{-1}M^{-1}$), the assay can be performed by either method. Resorufin is unstable in the presence of thiols such as DTT or 2-mercaptoethanol and is pH dependant, therefore the reaction was carried out at pH 7.5.

2.3.3.1 Reagents and Preparation

The kit contained the following reagents, which were prepared on opening the kit:

Amplex red reagent (Molecular Weight = 257): One vial of Amplex red was dissolved in 100 µL of the supplied Dimethyl sulfoxide **(**DMSO) and this stock solution was stored at -20°C in the dark when not in use.

Dimethyl sulfoxide (DMSO), anhydrous: This reagent was used to dilute the Amplex Red reagent and stored dessicated at room temperature.

Horseradish Peroxidase (HRP): Each kit contained 20U of HRP and a 100U/mL stock solution was prepared by dissolving the contents of the HRP vial in 200 µL of 1X reaction buffer. The reagent was divided into aliquots and stored at -20° C.

Hydrogen Peroxide (H_2O_2**,** Molecular Weight = 34), a ~3% solution: A 20 mM solution of H_2O_2 was prepared by diluting the appropriate ~3% solution with distilled H_2O.

5x Reaction Buffer: The reaction buffer consisted of 0.5M Tris HCl at pH 7.5. A 1X working stock was prepared by adding 1 mL of 5X reaction buffer to 4 mL of distilled H_2O and stored at 6°C.

Xanthine Oxidase (XO) from buttermilk: Each kit contained 1U of xanthine oxidase and a xanthine oxidase stock solution was prepared by dissolving the contents of the vial in 100 µL distilled H_2O and stored in aliquots at -20°C.

Hypoxanthine: The kit contained a vial of a 20 mM solution in 40 mM NaOH, which was used for the preparation of hypoxanthine standard curve.

Xanthine: The kit contained a vial of a 20 mM solution in 40 mM NaOH, which was used for the preparation of xanthine standard curve.

Each kit provided enough materials for ~400 assays to be done in a total volume of 100 µL per assay in a 200 µL Polymerase Chain Reaction tube measured in a 100 µL cuvette.

2.3.3.2 Procedure

The reagents prepared from the kit were allowed to come to room temperature after being removed from the -20° C freezer.

Hypoxanthine standard curve: A hypoxanthine standard curve was created by diluting the 20 mM hypoxanthine in 1X reaction buffer and hypoxanthine concentrations between 0 to 100 µM were produced by the following combinations:

For 100 µM – 10 µL of 20 mM of hypoxanthine + 1990 µL 1X reaction buffer

For 80 µM – 400 µL of 100 µM of hypoxanthine + 100 µL 1X reaction buffer

For 60 µM – 300 µL of 100µM of hypoxanthine + 200 µL 1X reaction buffer

For 40 µM – 200 µL of 100 µM of hypoxanthine + 300 µL 1X reaction buffer

For 20 µM – 100 µL of 100 µM of hypoxanthine + 400 µL 1X reaction buffer

For 0µM (blank) – 1X reaction buffer was used.

The vitreous sample was diluted 1/10 with 1X reaction buffer. To the 50 µL of the 1/10 diluted vitreous sample was further added 50 µL of the Amplex red reagent to make a total of 100µL in the sample tube. Each sample tube had a corresponding duplicate for the purpose of reproducibility. A positive control was prepared by diluting the 20 mM working solution of H_2O_2 with 20 µM of 1X Reaction Buffer. This was done by diluting 10µL of H_2O_2 with 990 µL of 1X reaction buffer to get a 200 µM solution. This 200 µM solution was diluted by adding 50 µL of it with 950 µL of 1X reaction buffer to get a 10 µM solution. This resultant solution was run as a sample for a positive control in the assay reaction.

50 µL of each sample/standard/blank to be analyzed was pipetted into separate eppy tubes. A working solution of Amplex Red reagent containing 0.4 U/mL HRP and 40 U/mL XO was prepared by mixing 15 µL Amplex Red stock solution, 6 µL HRP stock solution, 6 µL of the XO stock solution and 1473 µL of 1X Reaction Buffer. The reactions were begun by adding 50 µL of the Amplex Red reagent to each of the standard/ sample tubes. The tubes were mixed well and centrifuged briefly to ensure adequate mixing. The reaction tubes were incubated at 37 °C in the oven for approximately 30 minutes and centrifuged briefly to compensate for the minimal evaporation in the incubator, before being read on the spectrophotometer. The sample tray was adequately covered with tin foil, as the reagent in the assay is light sensitive. The absorbance of the samples was measured at 560 nm, using the non-hypoxanthine blank to zero the spectrophotometer. Since the assay reaction was

continuous, the spectrophotometer was blanked with a blank every 10 samples, thus preventing the skewing of the results due to the delays between the original control reading and the sample reading. The measurements of the hypoxanthine control samples were used to create a standard curve using Microsoft Excel® and this curve was used to determine the sample concentrations of hypoxanthine. This was achieved by plotting the mean absorbance of the sample tube on the *y*- axis and then dropping a perpendicular line from the standard curve down towards the *x*-axis. The resulting value as plotted on the *x*- axis was later multiplied by the dilution factor (i.e. 10) to obtain the appropriate measurement for hypoxanthine and xanthine levels in the respective vitreous sample.

2.3.4 Measurement of Lipid Hydroperoxides

The biochemical analyses for measuring vitreous lipid hydroperoxides were carried out using the Ferrous Oxidation in Xylenol assay version 2 as described by Nourooz-Zadeh et al. (Nourooz Zadeh et al. 1994). The method was originally designed to measure plasma hydroperoxides. This assay is based on the selective oxidation of ferrous to ferric ions by hydroperoxides under acidic conditions. The ferric ions generated by the ROOHs in the assay are complexed by the ferric indicator, xylenol orange, generating a blue complex with an absorbance maximum of 550 to 600 nm. For plasma samples, the signal generated using the ferrous oxidation in xylenol orange (FOX) assay is authenticated by prior reduction of plasma ROOHs with triphenylphosphine (TPP), thereby generating an internal control. This method does not require the plasma lipid extraction step and is capable of measuring all classes of hydroperoxides present in plasma with precision

as compared to High Performance Liquid Chromatography (HPLC)/ chemiluminescence methods. In the present study, the method was adopted to measure vitreous lipid hydroperoxide levels.

2.3.4.1 Reagents

Ammonium ferrous sulphate and Concentrated Sulfuric acid (H_2SO_4) (BDH Chemicals, Toronto, ON); hydrogen peroxide (H_2O_2) and HPLC grade methanol (EMD Biosciences, Gibbstown, NJ); butylated hydroxytoluene (BHT), xylenol orange [o-cresolsulfonphthalein-3,3'-bis(methyliminodiacetic acid) sodium salt], and Triphenylphosphine (TPP) (Sigma- Aldrich Inc., Oakville, ON) were the reagents employed in the assay.

2.3.4.2 Reagent Preparation

FOX 2 reagent: The reagent was prepared by dissolving 98 mg of ferrous ammonium sulphate in 100 mL of 250 mmol H_2SO_4. The H_2SO_4 was prepared beforehand by diluting 1.38 mL of concentrated H_2SO_4 with 100 mL of distilled H_2O. 176 mg BHT and 15.2 mg xylenol orange were dissolved in 150 mL of HPLC grade methanol. Once dissolved, 20 mL of ferrous ammonium sulphate solution was added to this mixture and was brought to 200 mL with the addition of methanol. The final solution had 250 μM ammonium ferrous sulphate, 100 μM xylenol orange, 25 mM H_2SO_4 and 4 mM BHT in 90% vol/vol methanol. This solution was routinely calibrated against H_2O_2 solutions of known concentration with every use. The extinction co-efficient used was 3.86 X 10^4 $M^{-1}cm^{-1}$. Extinction co-efficients lower than 3.72 X 10^4 $M^{-1}cm^{-1}$ were not accepted.

TPP solution: A 10 mM TPP solution was prepared in HPLC grade methanol by dissolving 0.2623g of TPP in 100 mL methanol. This solution removes all hydroperoxides from an aliquot in the sample thus creating a blank for each sample in the assay. All the vitreous samples had varying levels of ferric ion in them which would react with the xylenol orange, thus requiring each sample to have its' own blank.

2.3.4.3 Procedure

Preparation of standards: 1 mL of 0, 5, 10, 20, 30 and 50 µmol/L solutions of hydrogen peroxide in distilled water were prepared to create the standard curve. According to Fisher scientific, 30% hydrogen peroxide is equal to 9.8 M H_2O_2 (or 9.8 mol/mL). Using this concentration as the guide, 10.2 mL of 30% hydrogen peroxide was diluted with 500 mL distilled H_2O to get a 200 mM solution. From the 200 mM solution, the 20 mM standard was prepared by adding 250 µL of 200 mM solution to 250 mL distilled H_2O for 200 µM. The 200 µM solution was diluted as follows to prepare the standards:

For 100 µM: 3 mL of 200µM solution was diluted with 3 mL of water and mixed adequately

For 50 µM: 1 mL of 200 µM solution was diluted with 3 mL of water and mixed adequately.

For 30 µM: 1.5 mL of 100 µM solution was diluted with 3.5 mL of water and mixed adequately.

For 20 µM: 500µL of 200µM solution was diluted with 4.5 mL of water and mixed adequately.

For 10 µM: 500 µL of 100 µM solution was diluted with 4.5 mL of water and mixed adequately.

For 5 µM: 1 mL of 20 µM solution was diluted with 3 mL of water and mixed adequately

Distilled H_2O for the blank (0 µM)

These standards were used immediately following preparation. 90 µL of hydrogen peroxide standards that were prepared were aliquoted in three tubes for each standard and 10 µL of methanol was added to each of the standard tubes. Further, 900 µL of FOX 2 reagent was added to each tube and the sample incubated at room temperature for 30 minutes after adequate vortexing of the tubes. The absorbance of standard was measured at 560 nm using the UNICAM spectrophotometer and the measurements were used to create a graph and calculate the values of test samples.

Preparation of sample: 300 µL of vitreous humor sample was aliquoted into separate tube after centrifugation at 2050 X g for 10 minutes. The vitreous humor sample was adequately diluted in a 1:1 ratio with distilled water. Therefore, 280 µL of vitreous humor was diluted with 280 µL of distilled H_2O. This dilution is necessary to obtain accurate results as the absorbance of an undiluted vitreous sample falls outside the standard curve, as the curve becomes non-linear at ~30-50 µM.

Measurement of hydroperoxides: 90 µL of vitreous sample were transferred to 6 microcentrifuge tubes (5 mL). For the first three sample tubes, 10 µL of 10 mmol/L TPP in methanol solution was added to reduce ROOHs, thereby generating three blank tubes of test samples. 10 µL of methanol was added to the remaining three

tubes and these constituted the test samples measured for the hydroperoxide content. All the tubes were then vortexed and incubated at room temperature for 30 minutes. After 30 minutes of incubation, 900 μL of FOX 2 reagent was added to all the 6 tubes. The tubes were adequately mixed and the samples were further incubated at room temperature for another 30 minutes and then centrifuged at 2400 X g for 10 minutes to pellet any particulate matter that would alter the reading and also clog up the sample sipper of the spectrophotometer. Absorbance of the supernatant was measured at 560 nm using the UNICAM spectrophotometer.

Calculations: The standards were used to create a graph and calculate the values of test samples. The results were multiplied by a factor of 2 to correct for the dilution. The hydroperoxides in the sample is calculated as a function of the mean absorbance of the difference in absorbance between the samples with and without elimination of lipid peroxides by TPP. The Microsoft Excel® spreadsheet was used to average the values of three blanks (samples with TPP in methanol) and test samples (samples with methanol), and subtract the two values to obtain the average absorbance of the hydroperoxide for each sample. The difference in the mean absorbance of each sample at 560 nm was used to calculate the hydroperoxide content of the sample. The absorbance values from the hydrogen peroxide standards were used create a standard curve and to add a trend line to the graph. This graph and the equation of the trend line of the graph were used to calculate the concentration of hydroperoxides in each sample.

2.4 STATISTICAL ANALYSES

The statistical analyses for the data were carried out using the Statistical Package for Social Sciences (SPSS®) for Windows™ Version 13.0.

2.4.1 Between-Eye Differences

Paired samples T test and paired samples correlation analyses was employed for the statistical evaluation of the vitreous biochemical constituent concentration differences between the same pair of eyes.

2.4.2 Correlation of Vitreous Constituents and PMI

Linear regression correlation analyses were used to establish the correlation between individual vitreous biochemical constituents and the PMI.

2.4.2.1 Linear Regression Equation and Formulae

The linear regression formulae to estimate PMI using individual vitreous biochemical constituents were derived by the following method:

(i) Knowing the measured concentrations of individual vitreous biochemical constituents (mean value of right and left eye concentrations) and the actual PMI (hours), the following equation was formulated:

$$y = ax + b \quad \text{.....(2.1)}$$

where 'y' is mean of the right and left eye vitreous biochemical constituent concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line.

(ii) Estimated PMI (hours) was calculated using equation 2.1:

$$x = ((y - b) / a) \quad \text{.....(2.2)}$$

(iii) Using the estimated PMI (hours) x in equation 2.2 as the dependent variable and the individual vitreous biochemical constituent concentration as the independent variable, the following formula was derived:

Estimated PMI = $ß_0$ + $ß_1$ (Mean of the individual biochemical constituent concentration) ……...(2.3)

Where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for the individual vitreous biochemical constituent.

2.4.2.2 Comparison of Linear Regression Formulae

Paired T test were used for the statistical comparison of linear regression formulae derived for individual constituent or in comparison of the formulae derived in the present study with the previously reported formulae based on the specific vitreous biochemical constituent.

2.4.2.3 Correlation of a Pair of Vitreous Constituents and PMI

Multiple regression analyses was employed to study the significance of using two vitreous constituents in the same linear regression model to estimate PMI.

2.4.3 Comparison of Antemortem Serum and Postmortem Vitreous Biochemistry

Linear regression correlation analyses were used to establish the statistical correlation between antemortem serum and postmortem vitreous biochemical concentrations.

2.4.4 Comparison of Biochemical Parameters in Diabetic and Non-Diabetic Subjects

Some of the cases were assigned to two diagnostic subgroups based on the definite presence or absence of an established diagnosis of diabetes mellitus in the subject's medical records. By this method, the first diabetic subgroup with a previous diagnosis of diabetes mellitus comprised of 18 subjects (Mean age ± SD, 58.80 ± 16.78) and the second non-diabetic subgroup comprised of 54 subjects (Mean age ± SD, 60.10 ± 17.61) in whom a diagnoses of diabetic mellitus was excluded. For the statistical analyses of the data, Mann-Whitney test were used to study and compare the mean differences in vitreous glucose, lactate, and lipid hydroperoxide concentrations in the two sub-groups.

2.5 ETHICAL APPROVAL

The experimental protocol was approved by the University Biomedical Research Ethics Board (EC # 2002-00724).

3.0 RESULTS

3.1 VITREOUS HUMOR BIOCHEMICAL CONCENTRATIONS

Table 1. summarizes the statistical parameters of vitreous biochemical concentrations ranges based upon the mean values of the right and left eye samples.

Sodium: The mean range of sodium concentration observed in the subjects varied between 131 mmol/L to 187.5 mmol/L (Mean ± SD, 159.02 ± 10.22; SEM, 1.02).

Potassium: The mean range of vitreous potassium concentration in the subjects varied between 6.2 mmol/L to 19.05 mmol/L (Mean±SD, 11.75 ± 3.42; SEM, 0.37).

Chloride: The mean range of chloride concentration observed in the subjects varied between 86.5 mmol/L to 136 mmol/L (Mean ± SD, 112.49 ± 11.18; SEM, 1.12).

Calcium: The mean range of calcium concentration observed in the subjects varied between 1.61 mmol/L to 3.09 mmol/L (Mean ± SD, 2.09 ± 0.28; SEM, 0.03).

Magnesium: The mean range of magnesium concentration observed in the subjects varied between 0.01 mmol/L to 1.15 mmol/L (Mean ± SD, 0.56 ± 0.23; SEM, 0.02).

Urea: The mean range of urea concentration observed in the subjects varied between 1.9 mmol/L to 58.5 mmol/L (Mean ± SD, 7.58 ± 8.11; SEM, 0.82).

Creatinine: The mean range of creatinine concentration observed in the subjects varied between 12 μmol/L to 578 μmol/L (Mean ± SD, 64.58 ± 69.77; SEM, 6.98).

Table. 1. The observed concentrations of various vitreous humor biochemical constituents studied.

Constituent	n	Concentration			
		Range	Mean	Std. Deviation	Std. Error Mean
Potassium (mmol/L)	85	6.2-19.05	11.75	3.42	0.37
Hypoxanthine (μmol/L)	96	100.5-798.5	308.79	134.12	13.69
Xanthine (μmol/L)	70	204.5-1632	580.46	226.96	27.13
Lactate (mmol/L)	77	0.1-22.85	10.52	5.92	0.67
Calcium (mmol/L)	102	1.61-3.09	2.09	0.28	0.03
Sodium (mmol/L)	100	131-187.5	159.02	10.22	1.02
Chloride (mmol/L)	100	86.5-136	112.49	11.18	1.12
Magnesium (mmol/L)	87	0.01-1.15	0.56	0.23	0.02
Urea (mmol/L)	98	1.9-58.5	7.58	8.11	0.82
Creatinine (μmol/L)	100	12-578	64.58	69.77	6.98
Glucose (mmol/L)	85	0.15-28.1	2.16	3.78	0.41
Osmolality (mmol/kg)	78	282.5-436	323.75	32.04	3.63
Lipid hydroperoxides (μmol/L)	79	0-84.31	17.83	15.57	1.75

Glucose: The mean range of glucose concentration observed in the subjects varied between 0.15 mmol/L to 28.1 mmol/L (Mean ± SD, 2.16 ± 3.78; SEM, 0.41).

Lactate: The mean range of lactate concentration observed in the subjects varied between 0.1 mmol/L to 22.85 mmol/L (Mean ± SD, 10.52 ± 5.92; SEM, 0.67).

Osmolality: The mean range of osmolality observed in the subjects varied between 282.5 mmol/kg to 436 mmol/kg (Mean ± SD, 323.75 ± 32.04; SEM, 3.63).

Hypoxanthine: The mean range of hypoxanthine concentration in the subjects varied between 100.5 to 798.5 µmol/L (Mean ± SD, 308.79 ± 134.12; SEM, 13.69).

Xanthine: The mean range of xanthine concentration in the subjects varied between 204.5 µmol/L to 1632 µmol/L (Mean ± SD, 580.46 ± 226.96; SEM, 27.13).

Lipid hydroperoxides: The mean range of lipid hydroperoxides in the subjects varied between 0 to 84.31 µmol/L (Mean ± SD, 17.83 ± 15.57; SEM, 1.75).

3.2 BETWEEN-EYE DIFFERENCES

The statistical parameters range, mean, SD and SEM for the between-eye differences observed for various vitreous biochemical constituents are summarized in Table. 2. The paired samples correlation for various vitreous biochemical constituents of the same pair of eyes are summarized in Table. 3.

Sodium: The mean vitreous sodium concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 1. It was observed that the between-eye difference for vitreous sodium varied between 0 to 50 mmol/L (Mean ± SD, 13.26 ± 12.07; SEM, 1.21). These between-eye differences for vitreous sodium were not statistically significant (P= 1.0). The right and left eye

Table. 2. Between-eye differences for the various vitreous humor biochemical constituents studied.

Constituent	**n**	**Between eye differences**				
		Range	**Mean**	**Std. Deviation**	**Std. Error Mean**	***P* value**
Potassium (mmol/L)	85	0-7.5	1.1	1.44	0.16	0.101
Hypoxanthine (μmol/L)	96	0-215	39.74	40.96	4.1	0.150
Xanthine (μmol/L)	70	0-430	74.18	76.69	8.91	0.904
Lactate (mmol/L)	77	0-15.4	3.61	3.6	0.41	0.889
Calcium (mmol/L)	102	0-1.56	0.26	0.27	0.03	0.134
Sodium (mmol/L)	100	0-50	13.26	12.07	1.21	1.0
Chloride (mmol/L)	100	0-54	9.63	9.17	0.92	0.183
Magnesium (mmol/L)	87	0-0.63	0.17	0.15	0.02	0.977
Urea (mmol/L)	98	0-10.4	1.39	1.84	0.19	0.529
Creatinine (μmol/L)	100	0-46	7.98	8.36	0.84	0.325
Glucose (mmol/L)	85	0-18.2	0.61	2.03	0.22	0.472
Osmolality (mmol/kg)	78	0-10	2.09	2.02	0.23	0.672
Lipid hydroperoxides (μmol/L)	79	0-87.94	12.47	15.74	1.76	0.494

Table. 3. Paired samples correlation for various vitreous humor biochemical constituents of same pair of eyes.

Constituent	**n**	**R**	***P* value**
Potassium	85	0.872	<0.0001
Hypoxanthine	96	0.912	<0.0001
Xanthine	70	0.889	<0.0001
Lactate	77	0.685	<0.0001
Calcium	102	0.400	<0.0001
Sodium	100	0.128	0.205
Chloride	100	0.494	<0.0001
Magnesium	87	0.613	<0.0001
Urea	98	0.962	<0.0001
Creatinine	100	0.989	<0.0001
Glucose	85	0.858	<0.0001
Osmolality	78	0.996	<0.0001
Lipid hydroperoxides	79	0.425	<0.0001

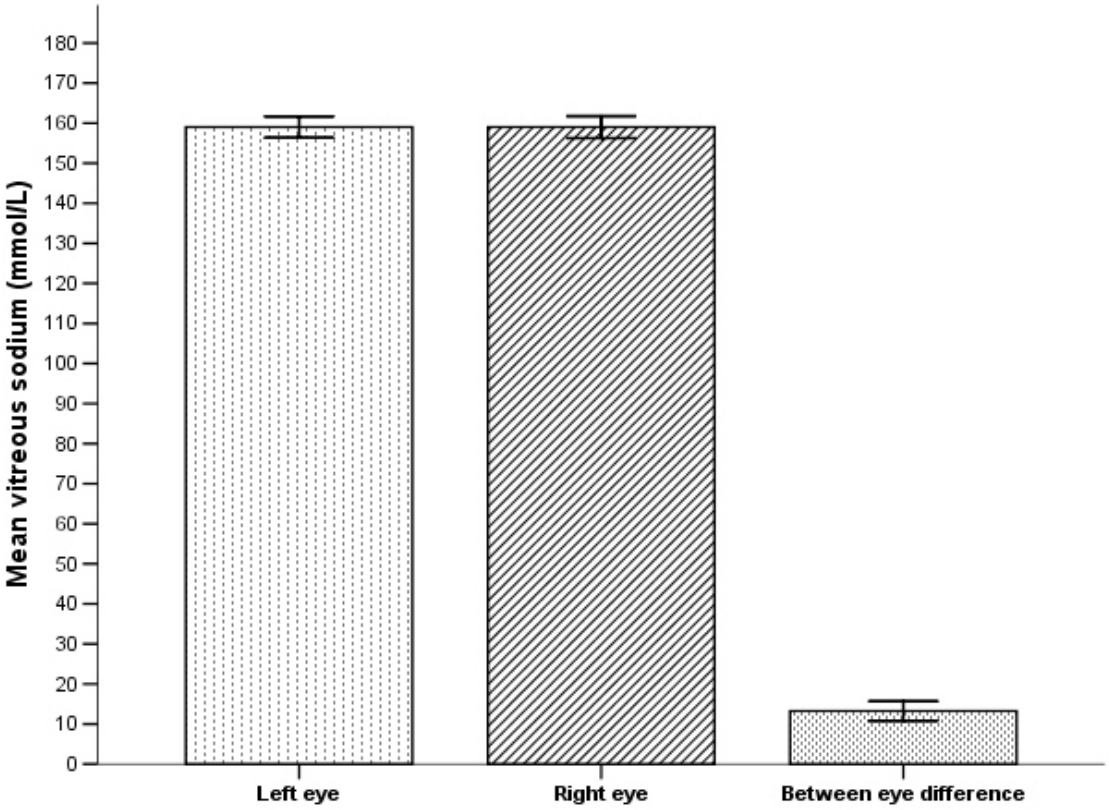

Fig. 1. Right and left eye vitreous humor sodium concentrations and the observed between-eye differences expressed as Mean ± SEM.

paired samples correlation was not found to be statistically significant for vitreous sodium concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 14.08 mmol/L (± SD, 13.25; SEM, 1.72). The paired samples correlation was observed to be insignificant even in the smaller subgroup of subjects with known PMI.

Potassium: The mean vitreous potassium concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 2. It was observed that the between-eye difference for vitreous potassium varied between 0 to 7.5 mmol/L (Mean ± SD, 1.1 ± 1.44; SEM, 0.16). These between-eye differences for vitreous potassium were not statistically significant ($P = 0.101$). The right and left eye paired samples correlation was highly significant ($P < 0.0001$) for vitreous potassium concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 1.07 mmol/L (± SD, 1.46; SEM, 0.19). A highly significant ($P < 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Chloride: The mean vitreous chloride concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 3. It was observed that the between-eye difference for vitreous chloride varied between 0 to 54 mmol/L (Mean ± SD, 9.63 ± 9.17; SEM, 0.92). These between-eye differences for vitreous chloride were not statistically significant ($P = 0.183$). The right and left eye paired samples correlation was highly significant ($P < 0.0001$) for vitreous chloride concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 9.85 mmol/L (± SD, 9.56; SEM,

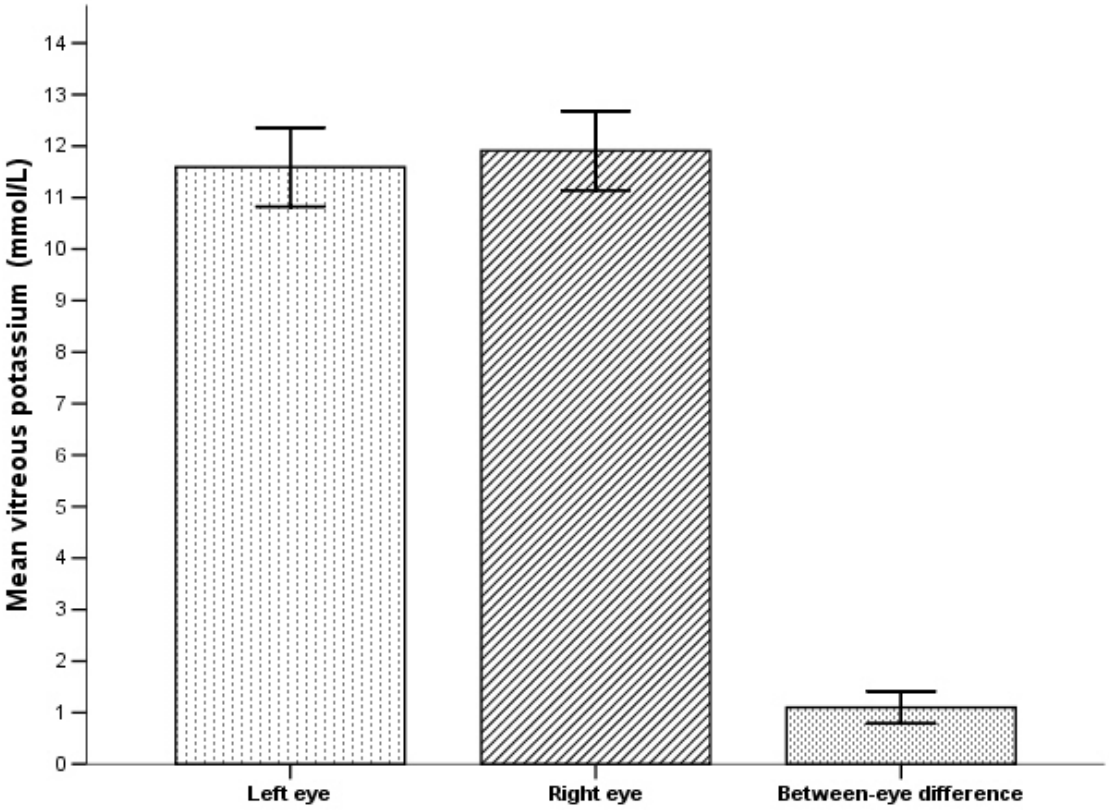

Fig. 2. Right and left eye vitreous humor potassium concentrations and the observed between-eye differences expressed as Mean ± SEM.

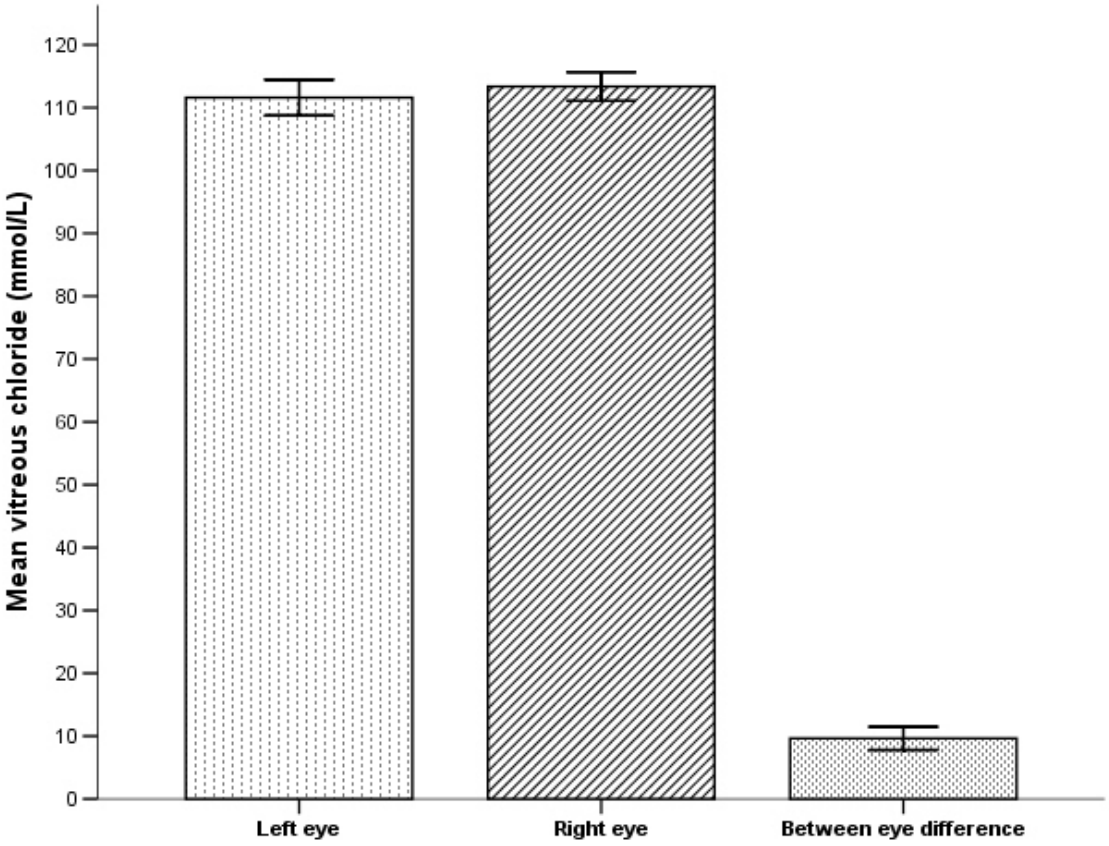

Fig. 3. Right and left eye vitreous humor chloride concentrations and the observed between-eye differences expressed as Mean ± SEM.

1.23). A highly significant ($P< 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Calcium: The mean vitreous calcium concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 4. It was observed that the between-eye difference for vitreous calcium varied between 0 to 1.56 mmol/L (Mean ± SD, 0.26 ± 0.27; SEM, 0.03). These between-eye differences for vitreous calcium were not statistically significant ($P= 0.134$). The right and left eye paired samples correlation was highly significant ($P< 0.0001$) for vitreous calcium concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 0.25 mmol/L (± SD, 0.21; SEM, 0.03). A highly significant ($P< 0.01$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Magnesium: It was observed that the between-eye difference for vitreous magnesium varied between 0 to 0.63 mmol/L (Mean ± SD, 0.17 ± 0.15; SEM, 0.02). These between-eye differences for vitreous magnesium were not statistically significant ($P= 0.977$). The right and left eye paired samples correlation was highly significant ($P< 0.0001$) for vitreous magnesium concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 0.16 mmol/L (± SD, 0.16; SEM, 0.02). A highly significant ($P< 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Urea: The mean vitreous urea concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 5. It was observed that the

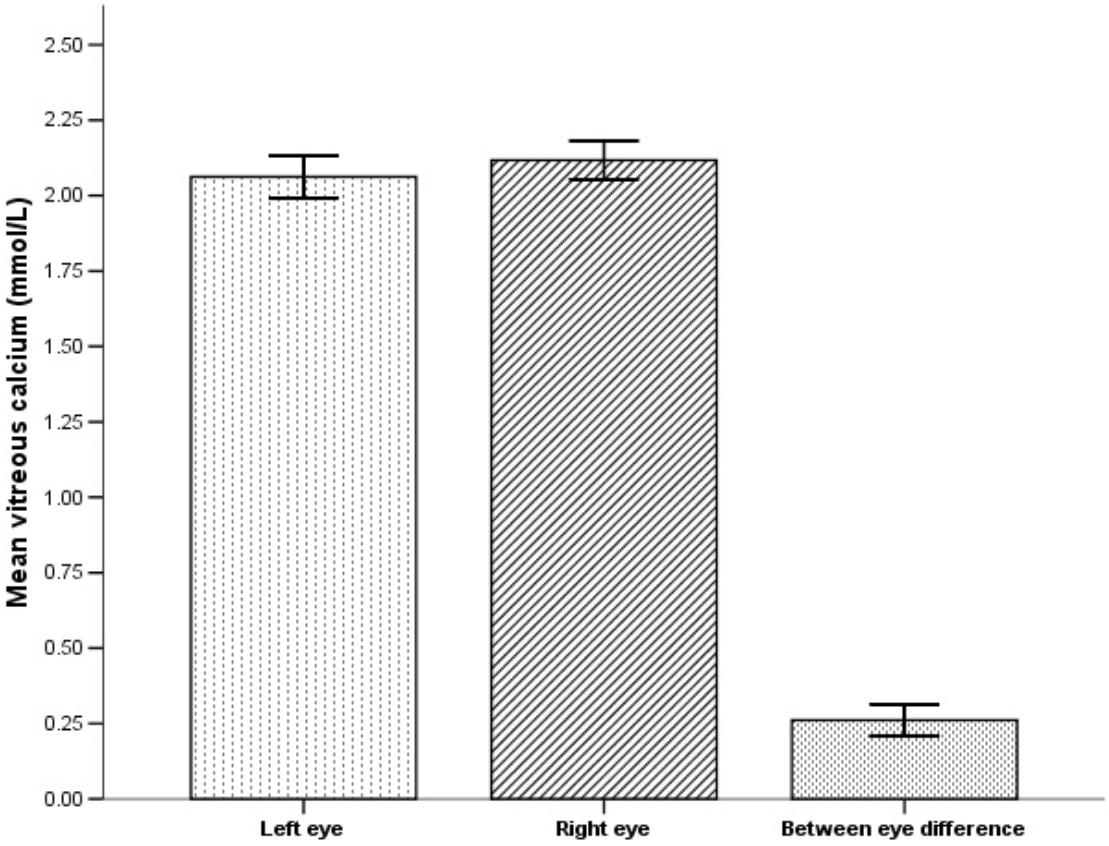

Fig. 4. Right and left eye vitreous humor calcium concentrations and the observed between eye differences expressed as Mean ± SEM.

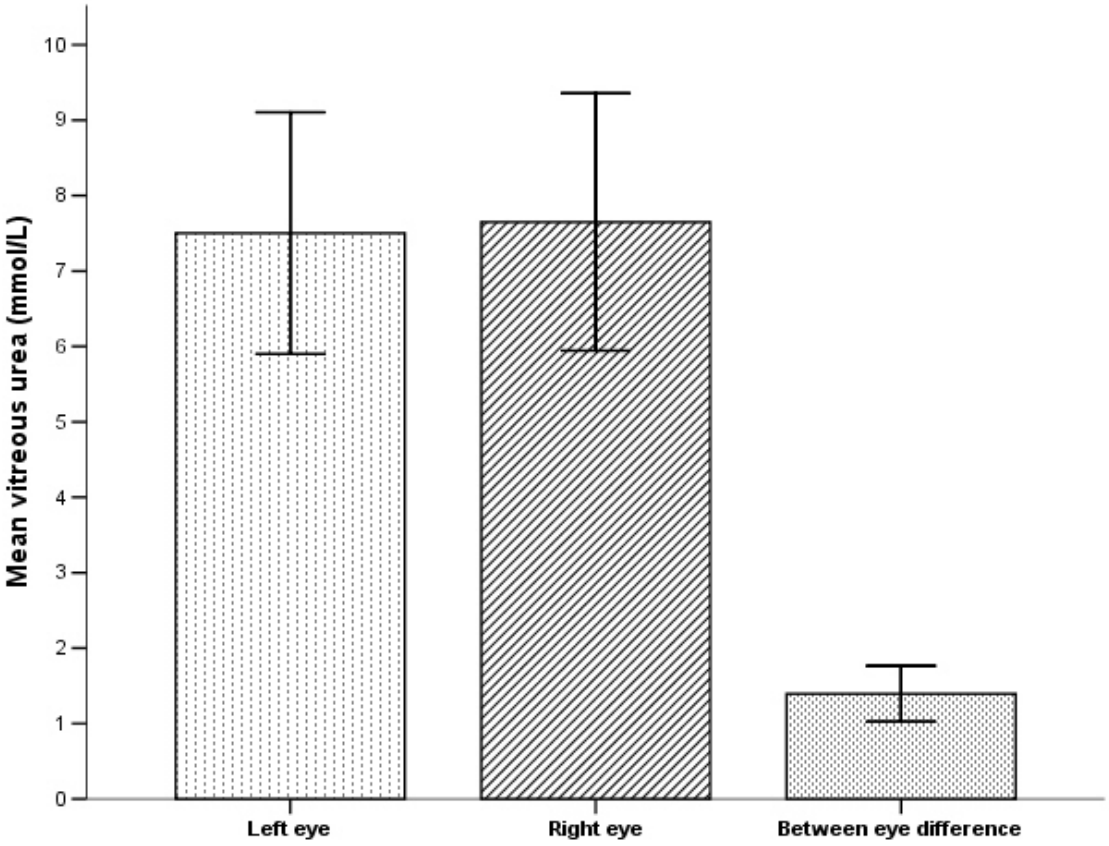

Fig. 5. Right and left eye vitreous humor urea concentrations and the observed between eye differences expressed as Mean ± SEM.

between-eye difference for vitreous urea varied between 0 to 10.4 mmol/L (Mean ± SD, 1.39 ± 1.84; SEM, 0.19). These between-eye differences for vitreous urea were not statistically significant (P= 0.529). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous urea concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 1.21 mmol/L (± SD, 1.53; SEM, 0.2). A highly significant (P< 0.0001) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Creatinine: The mean vitreous creatinine concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 6. It was observed that the between-eye difference for vitreous creatinine varied between 0 to 46 μmol/L (Mean ± SD, 7.98 ± 8.36; SEM, 0.84). These between-eye differences for vitreous creatinine were not statistically significant (P= 0.325). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous creatinine concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 7.42 μmol/L (± SD, 7.46; SEM, 0.97). A highly significant (P< 0.0001) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Glucose: It was observed that the between-eye difference for vitreous glucose varied between 0 to 18.2 mmol/L (Mean ± SD, 0.61 ± 2.03; SEM, 0.22). These between-eye differences for vitreous glucose were not statistically significant (P= 0.472). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous glucose concentrations. In the smaller subgroup of subjects

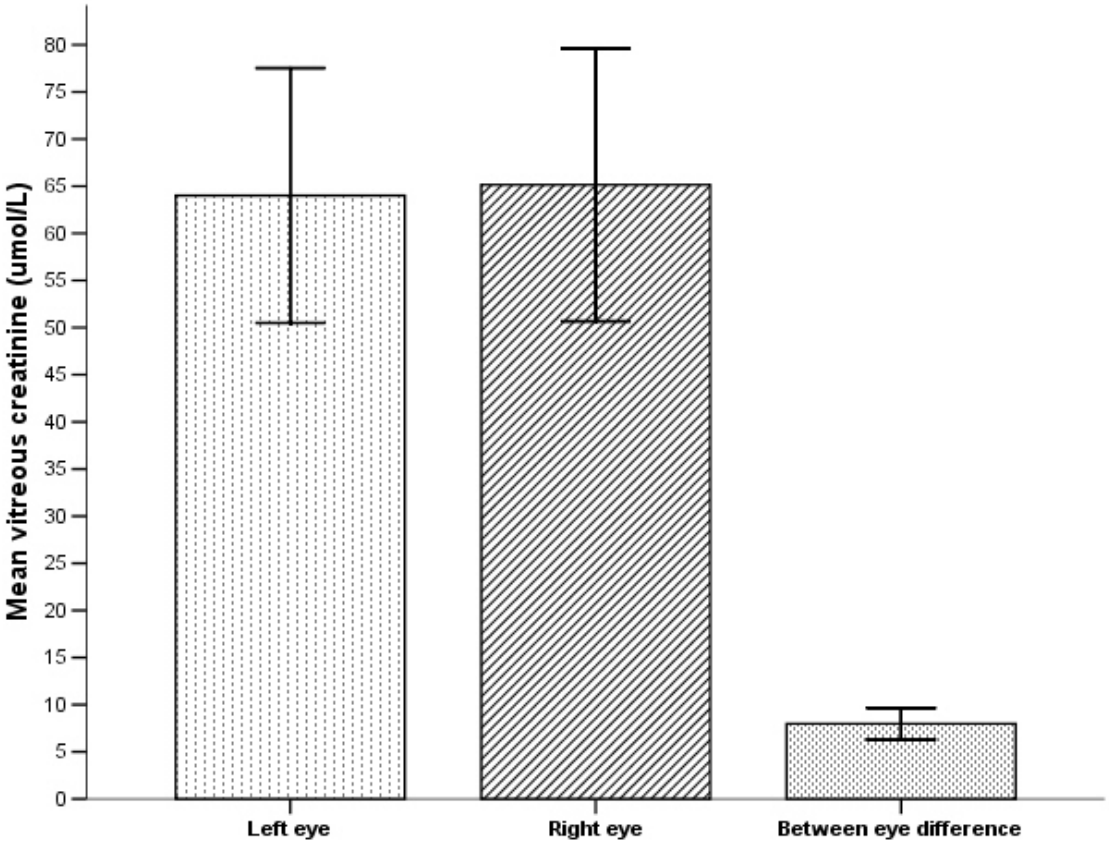

Fig. 6. Right and left eye vitreous humor creatinine concentrations and the observed between eye differences expressed as Mean ± SEM.

with known PMI, similar results were obtained with mean between eye variations of 0.43 mmol/L (± SD, 0.72; SEM, 0.1). A highly significant ($P< 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Lactate: The mean vitreous lactate concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 7. It was observed that the between-eye difference for vitreous lactate varied between 0 to 15.4 mmol/L (Mean ± SD, 3.61 ± 3.6; SEM, 0.41). These between-eye differences for vitreous lactate were not statistically significant ($P= 0.889$). The right and left eye paired samples correlation was highly significant ($P< 0.0001$) for vitreous lactate concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 3.16 mmol/L (± SD, 3.28; SEM, 0.48). A highly significant ($P< 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Osmolality: It was observed that the between-eye difference for vitreous osmolality varied between 0 to 10 mmol/kg (Mean ± SD, 2.09 ± 2.02; SEM, 0.23). These between-eye differences for vitreous osmolality were not statistically significant ($P= 0.672$). The right and left eye paired samples correlation was highly significant ($P< 0.0001$) for vitreous osmolality concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 1.9 mmol/kg (± SD, 1.87; SEM, 0.27). A highly significant ($P< 0.0001$) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

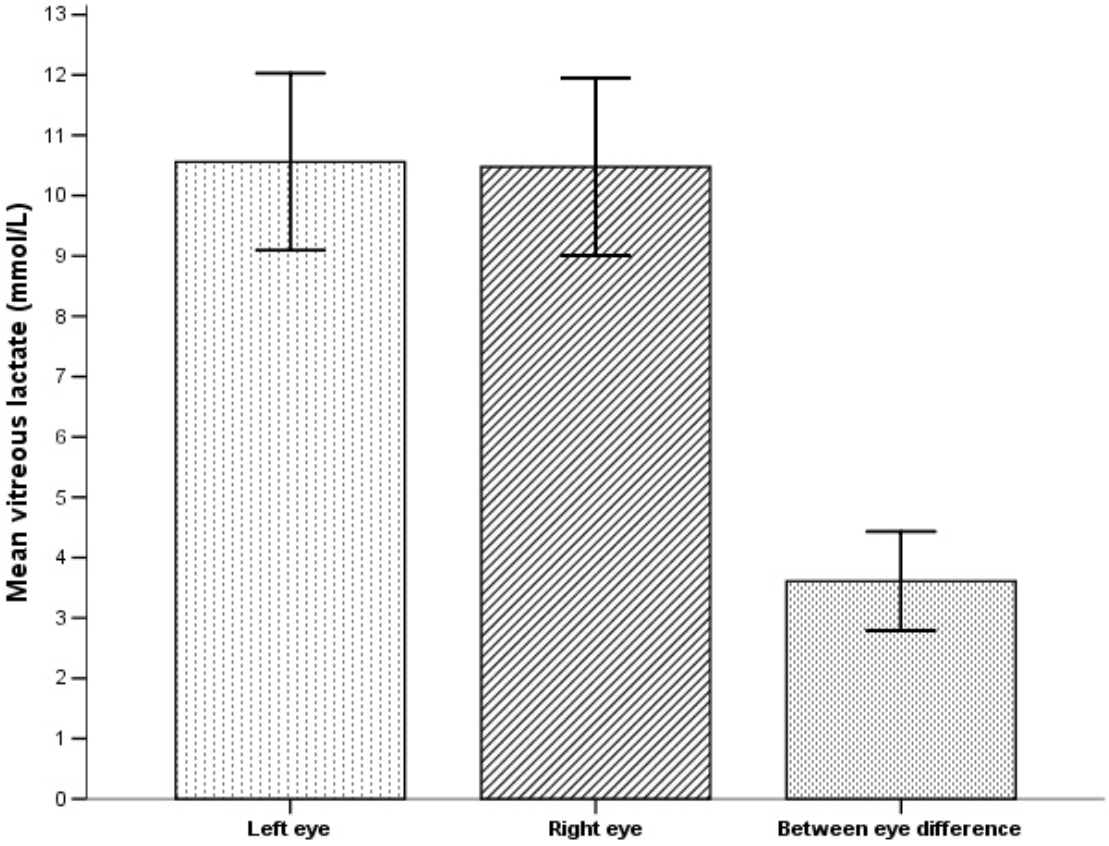

Fig. 7. Right and left eye vitreous humor lactate concentrations and the observed between eye differences expressed as Mean ± SEM.

Hypoxanthine: The mean vitreous hypoxanthine concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 8. It was observed that the between-eye difference for vitreous hypoxanthine varied between 0 to 215μmol/L (Mean ± SD, 39.74 ± 40.96; SEM, 4.1). These between-eye differences for vitreous hypoxanthine were not statistically significant (P= 0.150). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous hypoxanthine concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 31.65 μmol/L (± SD, 28.42; SEM, 3.76). A highly significant (P< 0.0001) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Xanthine: The mean vitreous xanthine concentrations for the right and left eyes along with their mean between-eye differences are shown in Fig. 9. It was observed that the between-eye difference for vitreous xanthine varied between 0 to 430 μmol/L (Mean ± SD, 74.18 ± 76.69; SEM, 8.91). These between-eye differences for vitreous xanthine were not statistically significant (P= 0.904). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous xanthine concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 70 μmol/L (± SD, 65.05; SEM, 9.59). A highly significant (P< 0.0001) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

Lipid hydroperoxides: It was observed that the between-eye difference for vitreous lipid hydroperoxides varied between 0 to 87.94 μmol/L (Mean ± SD, 12.47 ± 15.74;

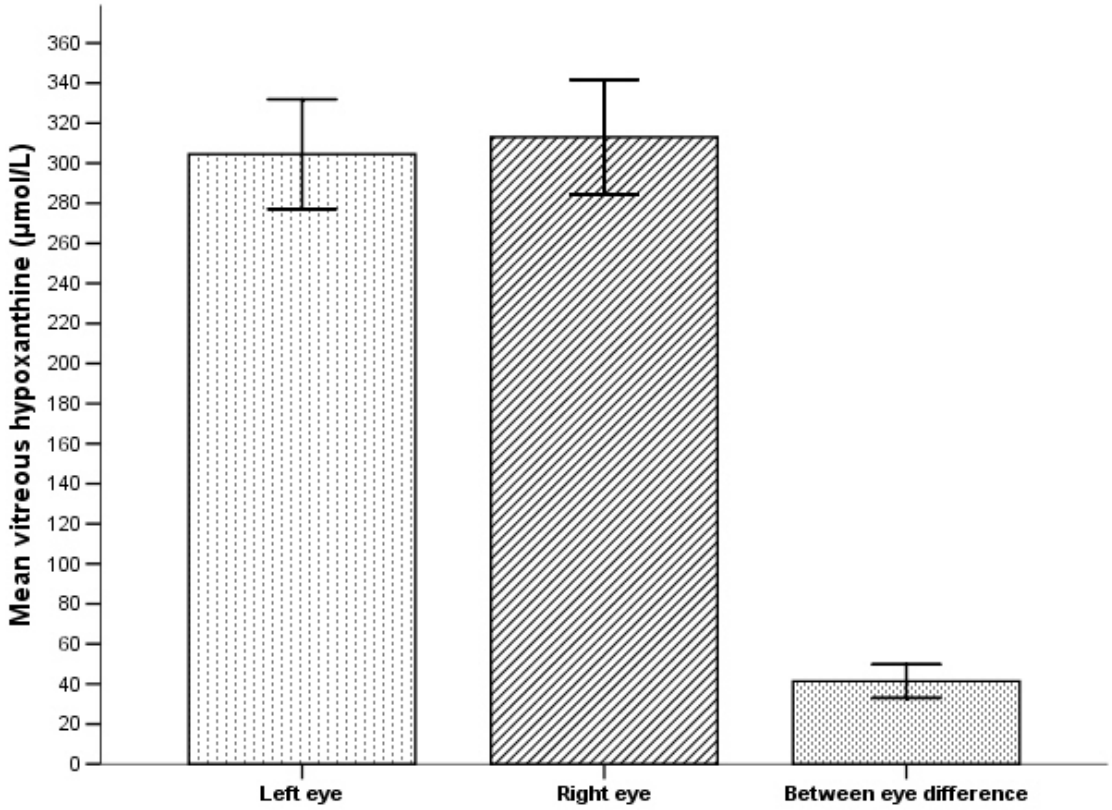

Fig. 8. Right and left eye vitreous humor hypoxanthine concentrations and the observed between eye differences expressed as Mean ± SEM.

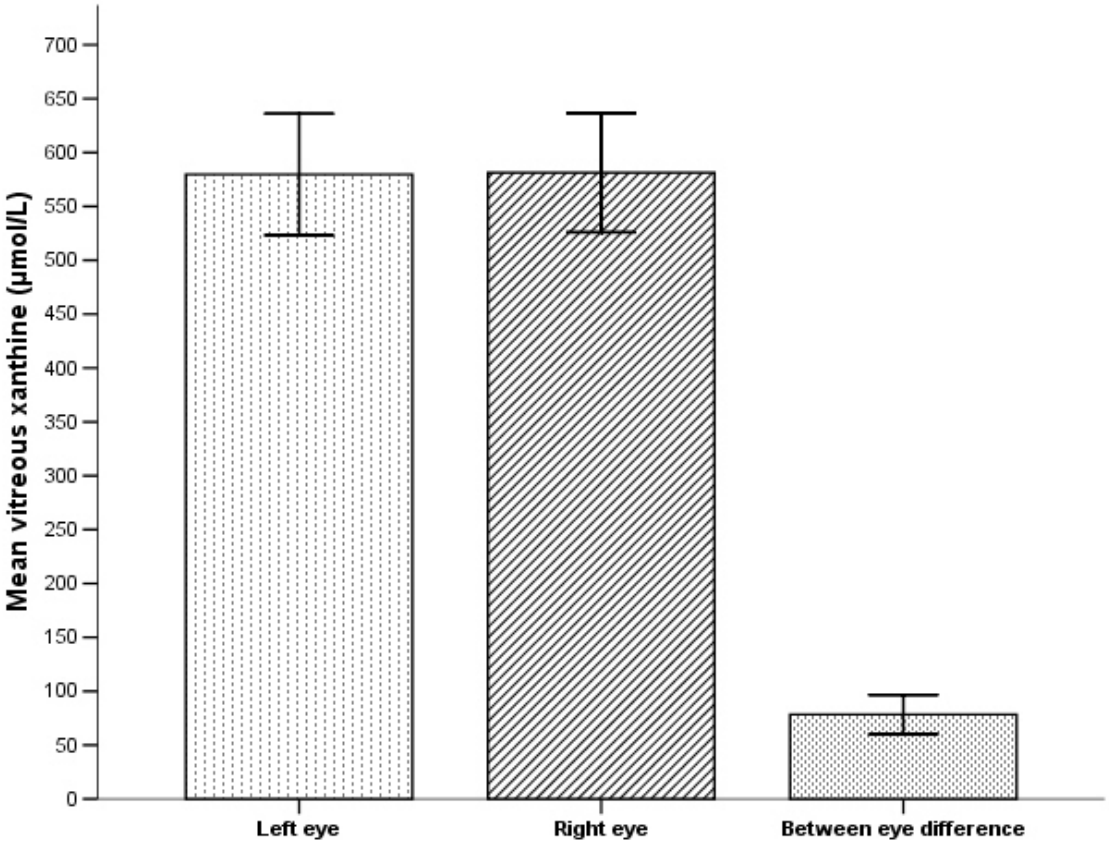

Fig. 9. Right and left eye vitreous humor xanthine concentrations and the observed between eye differences expressed as Mean ± SEM.

SEM, 1.76). These between-eye differences for vitreous lipid hydroperoxides were not statistically significant (P= 0.494). The right and left eye paired samples correlation was highly significant (P< 0.0001) for vitreous lipid hydroperoxide concentrations. In the smaller subgroup of subjects with known PMI, similar results were obtained with mean between eye variations of 15.2 µmol/L (± SD, 19.68; SEM, 2.97). A highly significant (P< 0.01) paired samples correlation was observed even in the smaller subgroup of subjects with known PMI.

3.3 VITREOUS BIOCHEMISTRY AND PMI CORRELATION

As a first step in determining a linear correlation between the various vitreous biochemical constituents and PMI, only subjects in whom the precise time of death was documented were included in this aspect of the study. Therefore, the study group was confined to 61 subjects, out of which 52 were hospital deaths and 9 were non-hospital deaths. Although the left and right eye aspirates were collected and analyzed separately, as reported earlier, none of the studied vitreous biochemical constituents exhibited any significant between eye differences. Therefore, for further statistical analysis of the data, only the mean values of both the eyes were considered. The linear regression correlation observed for the various vitreous analytes and PMI is tabulated in Table. 4.

Vitreous potassium and PMI: The linear rise of vitreous potassium against increasing PMI is represented in Fig. 10. The linear regression correlation of vitreous potassium and PMI was found to be highly significant (n, 58; R, 0.731; P< 0.0001). The right (R, 0.688; P< 0.0001) and left eyes (R, 0.722; P< 0.0001) vitreous potassium concentrations were also significantly correlated to PMI.

Table 4. The observed linear regression analyses correlation of the various vitreous analytes with PMI

Constituent	**n**	**R**	***P* value**
Potassium	58	0.731	<0.0001
Hypoxanthine	57	0.450	<0.0001
Xanthine	46	0.590	<0.0001
Lactate	47	0.508	<0.0001
Calcium	61	0.33	<0.01
Sodium	59	0.251	NS
Chloride	60	0.115	NS
Magnesium	52	0.101	NS
Urea	59	0.082	NS
Creatinine	59	0.065	NS
Glucose	54	0.119	NS
Osmolality	48	0.222	NS
Lipid hydroperoxides	44	0.043	NS

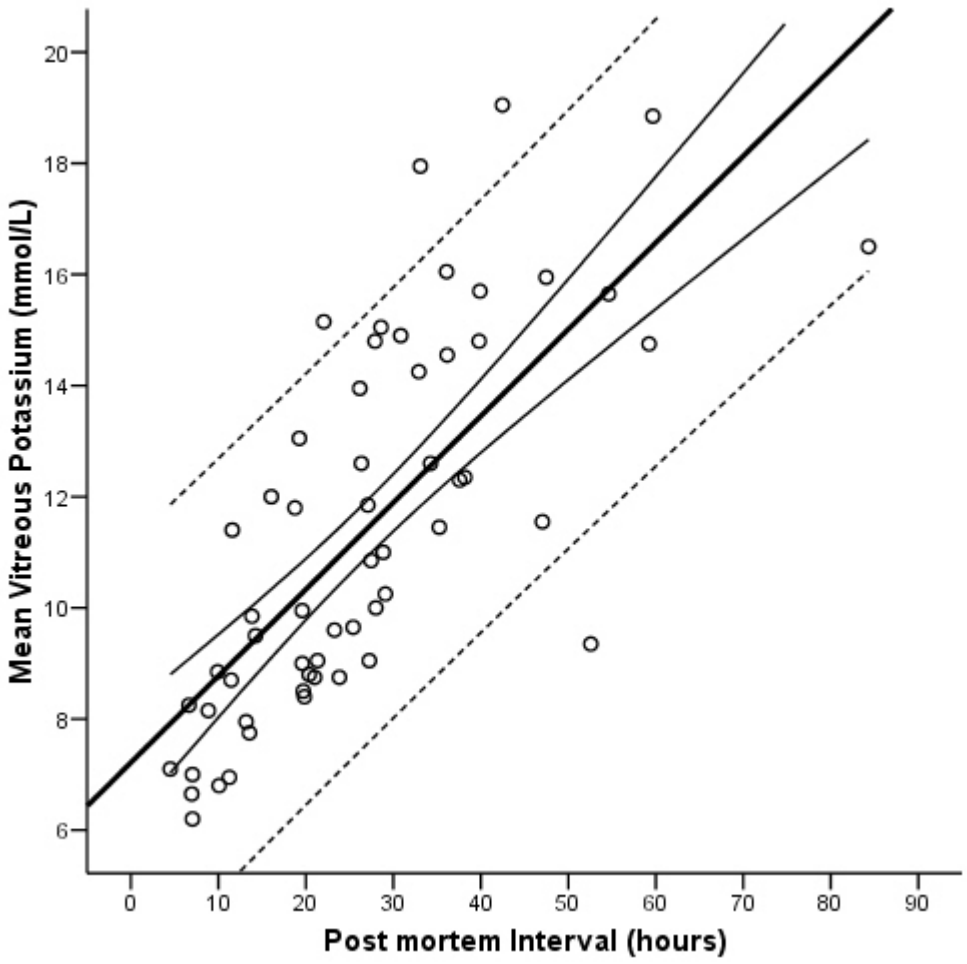

Fig. 10. The regression plot of mean vitreous humor potassium values plotted against the postmortem interval (PMI) in hours. Also shown are the regression line (━━ heavy solid) and the 95% Confidence Interval bands for the regression line (—— light solid) and the individual points (------ interrupted).

Vitreous hypoxanthine and PMI: The linear rise of vitreous hypoxanthine against increasing PMI is represented in Fig. 11. The linear regression correlation of vitreous hypoxanthine and PMI was found to be highly significant (n, 57; R, 0.450; *P*< 0.0001). The right (R, 0.411; *P*< 0.0001*)* and left eyes (R, 0.480; *P*< 0.0001) vitreous hypoxanthine concentrations were also significantly correlated to PMI.

Vitreous xanthine and PMI: The linear rise of vitreous xanthine against increasing PMI is represented in Fig. 12. The linear regression correlation of vitreous xanthine and PMI was found to be highly significant (n, 46; R, 0.590; *P*< 0.0001). The right (R, 0.638; *P*< 0.0001*)* and left eyes (R, 0.505; *P*< 0.0001) vitreous xanthine concentrations were also significantly correlated to PMI.

Vitreous lactate and PMI: The linear rise of vitreous lactate against increasing PMI is represented in Fig. 13. The linear regression correlation of vitreous lactate and PMI was found to be highly significant (n, 47; R, 0.508; *P*< 0.0001). The right (R, 0.442; *P*< 0.0001) and left eyes (R, 0.514; *P*< 0.0001) vitreous lactate concentrations were also significantly correlated to PMI.

Vitreous calcium and PMI: The linear rise of vitreous calcium against increasing PMI is represented in Fig. 14. The linear regression correlation of vitreous potassium and PMI was found to be highly significant (n, 61; R, 0.333; *P*< 0.01). The right (R, 0.277; *P*< 0.05*)* and left eyes (R, 0.280; *P*< 0.05) vitreous calcium concentrations were also significantly correlated to PMI.

Vitreous sodium and PMI: The linear regression correlation of vitreous sodium and PMI was found to be statistically insignificant (n, 59; R, 0.251; *P*, 0.06).

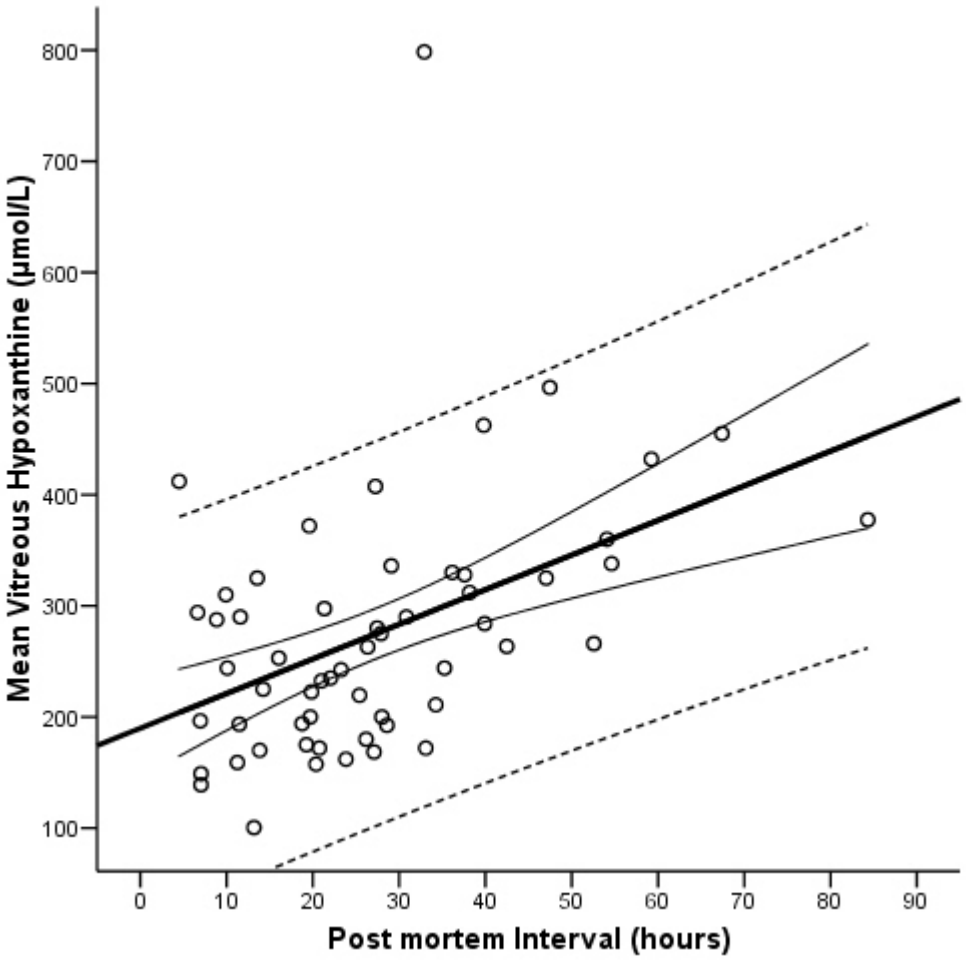

Fig. 11. The regression plot of mean vitreous humor hypoxanthine values plotted against the postmortem interval (PMI) in hours. Also shown are the regression line (━━ heavy solid) and the 95% Confidence Interval bands for the regression line (—— light solid) and the individual points (------ interrupted).

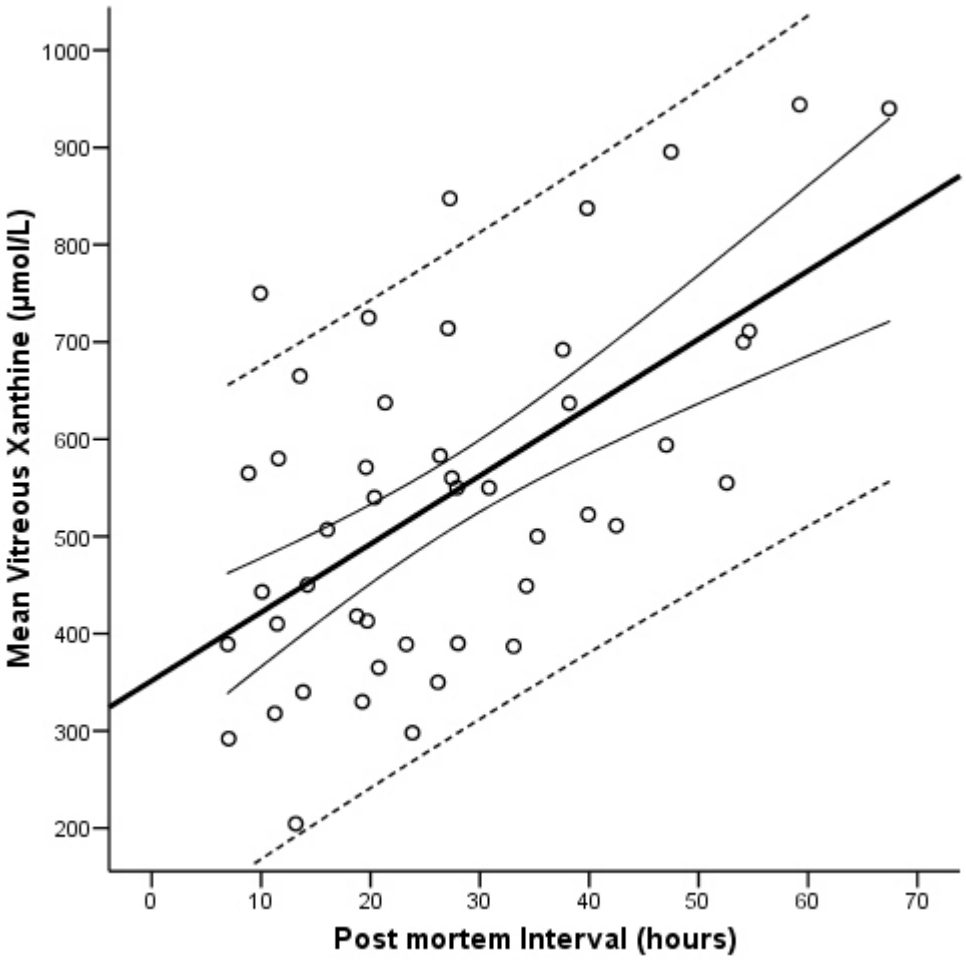

Fig. 12 The regression plot of mean vitreous humor xanthine values plotted against the postmortem interval (PMI) in hours. Also shown are the regression line (━━ heavy solid) and the 95% Confidence Interval bands for the regression line (—— light solid) and the individual points (------ interrupted).

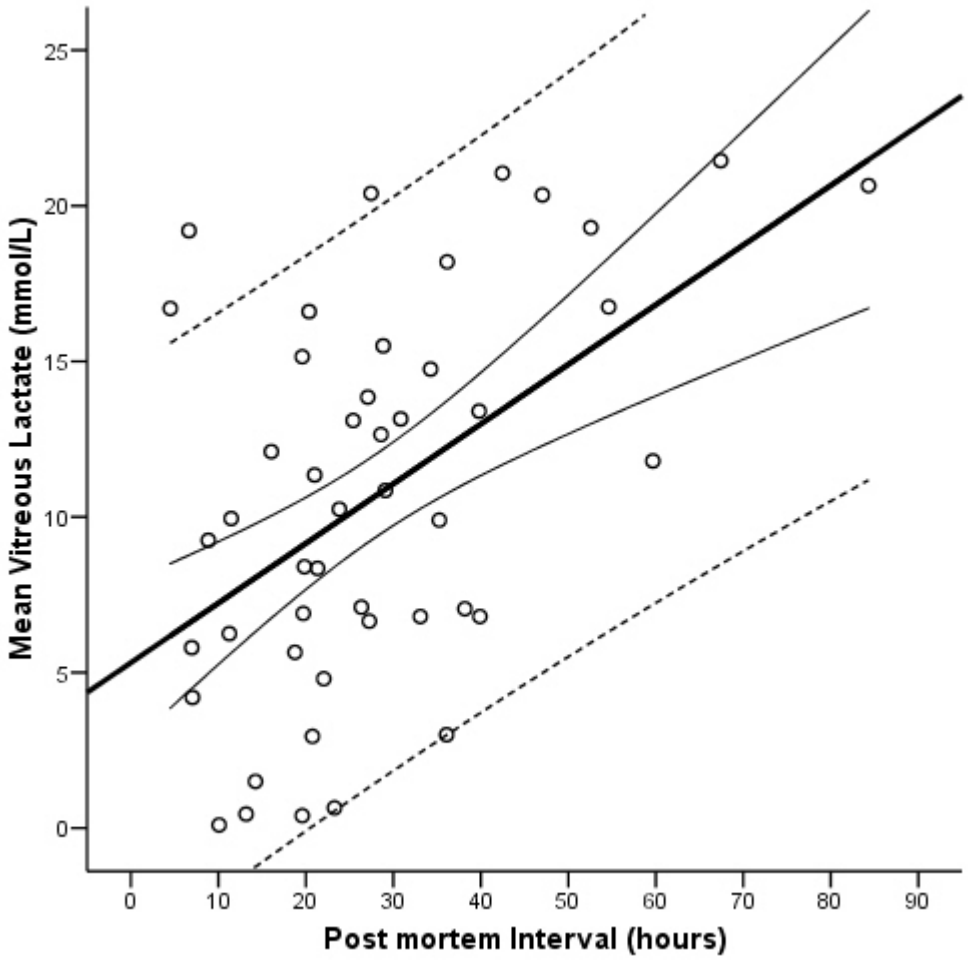

Fig. 13. The regression plot of mean vitreous humor lactate values plotted against the postmortem interval (PMI) in hours. Also shown are the regression line (━━ heavy solid) and the 95% Confidence Interval bands for the regression line (—— light solid) and the individual points (------ interrupted).

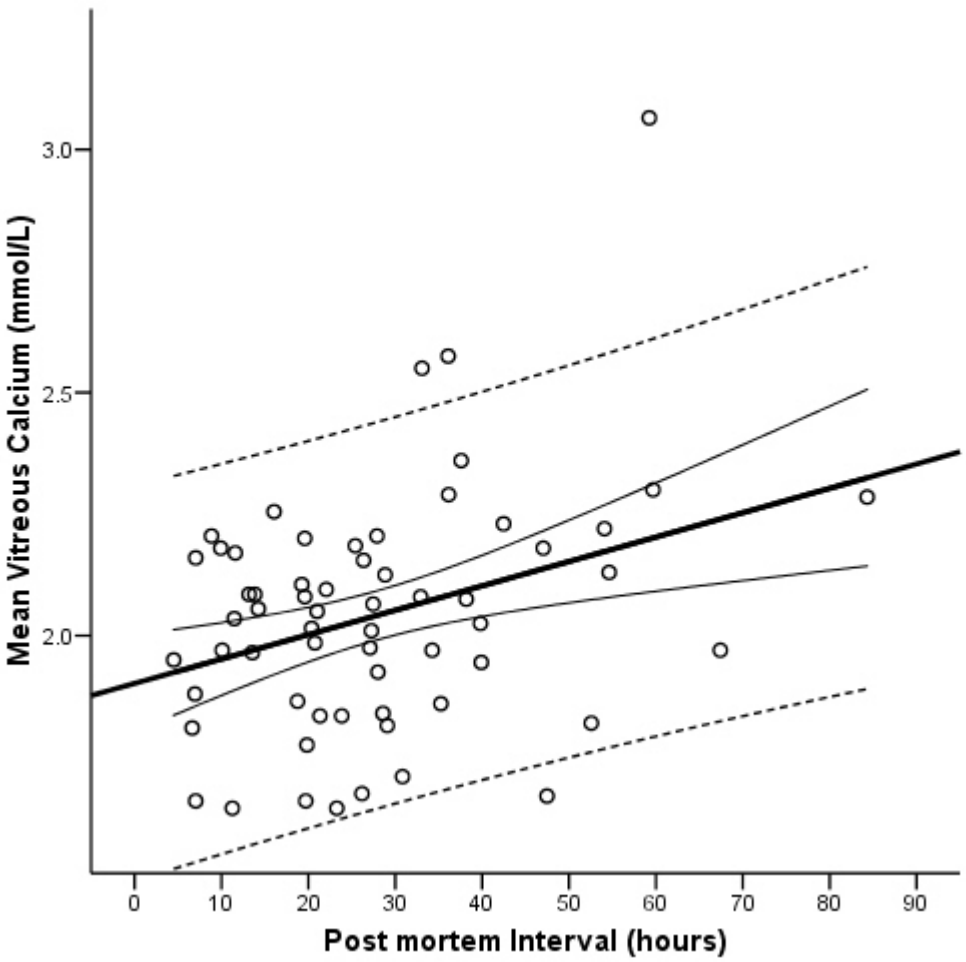

Fig. 14 The regression plot of mean vitreous humor calcium values plotted against the postmortem interval (PMI) in hours. Also shown are the regression line (▬▬ heavy solid) and the 95% Confidence Interval bands for the regression line (—— light solid) and the individual points (------ interrupted).

Similarly, the right and left eyes vitreous sodium concentrations were also not significantly correlated with PMI.

Vitreous chloride and PMI: The linear regression correlation of vitreous chloride and PMI was found to be statistically insignificant (n, 60; R, 0.115; *P,* 0.38). Similarly, the right and left eyes vitreous chloride concentrations were also not significantly correlated with PMI.

Vitreous magnesium and PMI: The linear regression correlation of vitreous magnesium and PMI was found to be statistically insignificant (n, 52; R, 0.101; *P,* 0.48). Similarly, the right and left eyes vitreous magnesium concentrations were also not significantly correlated with PMI.

Vitreous glucose and PMI: The linear regression correlation of vitreous glucose and PMI was found to be statistically insignificant (n, 54; R, 0.119; *P,* 0.39). Similarly, the right and left eyes vitreous glucose concentrations were also not significantly correlated with PMI.

Vitreous urea and PMI: The linear regression correlation of vitreous urea and PMI was found to be statistically insignificant (n, 59; R, 0.082; *P,* 0.54). Similarly, the right and left eyes vitreous urea concentrations were also not significantly correlated with PMI.

Vitreous creatinine and PMI: The linear regression correlation of vitreous creatinine and PMI was found to be statistically insignificant (n, 59; R, 0.065; *P,* 0.63). Similarly, the right and left eyes vitreous creatinine concentrations were also not significantly correlated with PMI.

Vitreous lipid hydroperoxides and PMI: The linear regression correlation of vitreous lipid hydroperoxides and PMI was found to be statistically insignificant (n, 44; R, 0.043; *P*, 0.78). Similarly, the right and left eyes vitreous lipid hydroperoxide concentrations were also not significantly correlated with PMI.

Vitreous osmolality and PMI: The linear regression correlation of vitreous osmolality and PMI was found to be statistically insignificant (n, 48; R, 0.222; *P*, 0.13). Similarly, the right and left eyes vitreous osmolality concentrations were also not significantly correlated with PMI.

3.3.1 Linear Regression Analyses and Proposed Formulae

3.3.1.1 Vitreous Humor Potassium

The mean values of the measured right and left eye vitreous potassium concentrations were used as the dependent variable to calculate the estimated PMI. The resulting linear regression equation in the form of $y = ax + b$ (where 'y' is mean of the right and left eye vitreous potassium concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line) was:

$$y = 0.16\,x + 7.22 \quad \text{……..……...(3.1)}$$

The corresponding formulae to estimate the PMI in the form of:

$$PMI = ß_0 + ß_1\,[Mean\ of\ the\ individual\ biochemical\ constituent\ concentration]$$

(where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for vitreous potassium) was:

$$Estimated\ PMI = 6.41\ (Potassium) - 46.25 \quad \text{…….(3.2)}$$

3.3.1.2 Vitreous Humor Hypoxanthine

The mean values of the measured right and left eye vitreous hypoxanthine concentrations were used as the dependent variable to calculate the estimated PMI. The resulting linear regression equation in the form of $y = ax + b$ (where 'y' is mean of the right and left eye vitreous hypoxanthine concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line) was:

$$y = 3.12x + 189.94 \quad \text{.....(3.3)}$$

The corresponding formulae to estimate the PMI in the form of:

$$PMI = ß_0 + ß_1 \text{ [Mean of the individual biochemical constituent concentration]}$$

(where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for vitreous hypoxanthine) was:

$$\text{Estimated PMI} = 0.32 \text{ (hypoxanthine)} - 60.94 \quad \text{.....(3.4)}$$

3.3.1.3 Vitreous Humor Xanthine

The mean values of the measured right and left eye vitreous xanthine concentrations were used as the dependent variable to calculate the estimated PMI. The resulting linear regression equation in the form of $y = ax + b$ (where 'y' is mean of the right and left eye vitreous xanthine concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line) was:

$$y = 7.02\,x + 351.72 \quad \text{.....(3.5)}$$

The corresponding formulae to estimate the PMI in the form of:

$$PMI = ß_0 + ß_1 \text{ [Mean of the individual biochemical constituent concentration]}$$

(where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for vitreous xanthine) was:

$$Estimated\ PMI = 0.14\ (Xanthine) - 50.08 \quad \text{……..(3.6)}$$

3.3.1.4 Vitreous Humor Lactate

The mean values of the measured right and left eye vitreous lactate concentrations were used as the dependent variable to calculate the estimated PMI. The resulting linear regression equation in the form of $y = ax + b$ (where 'y' is mean of the right and left eye vitreous lactate concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line) was:

$$y = 0.19\ x + 5.32 \quad \text{……..(3.7)}$$

The corresponding formulae to estimate the PMI in the form of:

$$PMI = ß_0 + ß_1\ [Mean\ of\ the\ individual\ biochemical\ constituent\ concentration]$$

(where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for vitreous lactate) was:

$$Estimated\ PMI = 5.21\ (Lactate) - 27.69 \quad \text{……(3.8)}$$

3.3.1.5 Vitreous Humor Calcium

The mean values of the measured right and left eye vitreous calcium concentrations were used as the dependent variable to calculate the estimated PMI. The resulting linear regression equation in the form of $y = ax + b$ (where 'y' is mean of the right and left eye vitreous calcium concentration; 'x' is actual PMI in hours; 'a' is the slope of regression line and 'b' is the intercept of the regression line) was:

$$y = 0.01\ x + 1.90 \quad \text{…… (3.9)}$$

The corresponding formulae to estimate the PMI in the form of:

$PMI = ß_0 + ß_1$ *[Mean of the individual biochemical constituent concentration]*

(where $ß_0$ is the estimated regression coefficient when no other variable is included in the model and $ß_1$ is the estimated regression coefficient for vitreous calcium) was:

Estimated PMI = 200 (calcium) – 380.4 …….(3.10)

3.3.2 Comparison of Paired Differences- Actual and Estimated PMI

The statistical parameters of paired comparison of actual PMI with the estimated PMI using the various derived formulae are summarized in Table. 5. It was found that the PMI estimations using the derived formulae based on potassium, hypoxanthine, xanthine, lactate and calcium were all significantly correlated with the actual PMI. The correlation was observed to be the highest for potassium based formulae (R, 0.731; $P< 0.0001$) and the least for the calcium-based formula (R, 0.333; $P< 0.01$). The remaining vitreous constituents, hypoxanthine (R, 0.450; $P< 0.0001$), xanthine (R, 0.590; $P< 0.0001$), and lactate (R, 0.508; $P< 0.0001$) were found to lie in between these two extremes.

3.3.3 Comparison of Derived Potassium Formula with other Reported Formulae

The comparison of various statistical parameters of paired differences between actual and estimated PMI obtained using the potassium based formulae derived from the present study and the previously reported formula by Sturner (1969), Madea et al. (1989) and James et al. (1997) are summarized in Table. 6.

Table. 5. Statistical comparison of differences between actual and estimated postmortem interval using the derived formulae

Constituent	**R**	**Mean**	**Std. Deviation**	**Std. Error Mean**	***P* value**
Potassium (mmol/L)	0.731	0.01	14.59	1.92	<0.0001
Hypoxanthine (µmol/L)	0.450	0.23	32.55	4.31	<0.0001
Xanthine (µmol/L)	0.590	1.29	20.40	3.01	<0.0001
Lactate (mmol/L)	0.508	0.02	27.99	4.08	<0.0001
Calcium (mmol/L)	0.333	0.05	46.80	5.99	<0.01

Table. 6. Comparison of statistical parameters of paired differences between actual and estimated post mortem interval using vitreous potassium formulae of various studies.

Study	**Slope**	**Mean**	**Std. Deviation**	**Std. Error Mean**	***P* value**
Sturner	0.14	15.47	16.35	2.15	<0.0001
Madea et al.	0.19	2.23	12.30	1.61	<0.0001
James et al.	0.23	4.06	11.08	1.45	<0.0001
Present	0.16	0.01	14.59	1.92	<0.0001

3.3.3.1 Comparison of Actual and Estimated PMI Using Derived Formula

As previously discussed, the potassium based formula derived from the data of the present study to estimate PMI is: *PMI = 6.41 (Potassium) – 46.25*. A highly significant correlationship ($P< 0.0001$) was noted when the actual PMI were compared with the estimated PMI using the derived formula. The standard deviation obtained between these two data sets was 14.59 hours.

3.3.3.2 Comparison of Actual and Estimated PMI Using Sturner Formula

When the potassium data obtained in the present study was substituted in the potassium based formula, *PMI = 7.14 (Potassium) – 39.1*, previously reported by Sturner (1963), the estimated PMI was significantly correlated ($P< 0.0001$) with the actual PMI with a standard deviation of 16.35 hours.

3.3.3.3 Comparison of Actual and Estimated PMI Using Madea et al. Formula

When the potassium data obtained in the present study was substituted in the potassium based formula, *PMI =5.26 (Potassium) – 30.9*, previously reported by Madea et al. (1989), the estimated PMI was significantly correlated ($P< 0.0001$) with the actual PMI with a standard deviation of 12.30 hours.

3.3.3.4 Comparison of Actual and Estimated PMI Using James et al. Formula

When the potassium data obtained in the present study was substituted in the potassium based formula, *PMI = 4.32 (Potassium) – 18.35*, previously reported by James et al. (1997), the estimated PMI was significantly correlated ($P< 0.0001$) with the actual PMI with a standard deviation of 11.08 hours with the actual PMI.

3.3.4 Comparison of Derived Hypoxanthine Formula with other Reported Formulae

The comparison of various statistical parameters of paired differences between actual and estimated PMI obtained using the hypoxanthine based formulae derived from the present study and the previously reported formula by James et al. (1997) and Munoz et al. (2002) are summarized in Table. 7.

3.3.4.1 Comparison of Actual and Estimated PMI Using Derived Formula

As previously discussed, the hypoxanthine based formula derived from the data of the present study to estimate PMI is: *PMI = 0.32 (Hypoxanthine) – 60.94*. A highly significant correlationship ($P< 0.0001$) was noted when the actual PMI were compared with the estimated PMI using the derived formula. The standard deviation obtained between these two data sets was 32.55 hours.

3.3.4.2 Comparison of Actual and Estimated PMI Using James et al. Formula

When the hypoxanthine data obtained in the present study was substituted in the hypoxanthine based formula, *PMI = 0.31 (Hypoxanthine) + 0.05*, previously reported by James et al. (1997), the estimated PMI was significantly correlated ($P< 0.0001$) with the actual PMI with a standard deviation of 31.23 hours.

3.3.4.3 Comparison of Actual and Estimated PMI Using Munoz et al. Formula

When the hypoxanthine data obtained in the present study was substituted in the hypoxanthine based formula, *PMI = 0.72 (Hypoxanthine) + 0.170*, previously reported by Munoz et al. (2002), the estimated PMI was significantly correlated ($P< 0.0001$) with the actual PMI with a standard deviation of 19.33 hours.

Table. 7. Comparison of statistical parameters of paired differences between actual and estimated postmortem interval using vitreous hypoxanthine formulae of various studies.

Study	**Slope**	**Std. Deviation**	**Std. Error Mean**	***P* value**
James et al.	3.2	31.23	4.07	<0.0001
Munoz et al.	3.01	19.33	2.52	<0.0001
Present	3.2	32.55	4.31	<0.0001

3.3.5 Multiple Regression Analysis with Potassium

Multiple regression equations using the individual vitreous biochemical constituents that had previously shown significant correlationship with PMI i.e. potassium, hypoxanthine, xanthine, lactate and calcium were derived. Since vitreous potassium exhibited the highest correlationship (R, 0.731; $P< 0.0001$) with PMI as compared to other vitreous constituents, potassium was used in conjunction with the remaining four constituents in the same linear regression model. A summary of the multiple regression correlationship of various constituents with potassium in the same model is provided in Table. 8.

3.3.5.1 Vitreous Potassium and Hypoxanthine

When vitreous hypoxanthine was included in the same regression model as vitreous potassium, vitreous hypoxanthine was observed to be an insignificant (P, 0.07) predictor of PMI in presence of vitreous potassium. The corresponding derived multiple regression was:

$$PMI = 2.61\ (Potassium) + 0.03\ (Hypoxanthine) - 15.66 \quad \text{.....(3.11)}$$

3.3.5.2 Vitreous Potassium and Xanthine

When vitreous xanthine was included in the same regression model as vitreous potassium, vitreous xanthine was observed to be a significant ($P< 0.01$) predictor of PMI in presence of vitreous potassium. The corresponding derived multiple regression was:

$$PMI = 2.61\ (Potassium) + 0.03\ (Xanthine) - 17.29 \quad \text{.....(3.12)}$$

Table. 8. Multiple regression analysis utilizing vitreous biochemical constituents in a single regression model along with vitreous potassium.

Constituent	**n**	**R (model)**	***P* value**
Potassium	54	0.728	<0.0001
Hypoxanthine			0.07
Potassium	43	0.765	<0.0001
Xanthine			<0.01
Potassium	45	0.774	<0.0001
Lactate			<0.05
Potassium	58	0.733	<0.0001
Calcium			0.57

3.3.5.3 Vitreous Potassium and Lactate

When vitreous lactate was included in the same regression model as vitreous potassium, vitreous lactate was observed to be a significant ($P< 0.05$) predictor of PMI in presence of vitreous potassium. The corresponding derived multiple regression was:

$$PMI = 2.98\ (Potassium) + 0.71\ (Lactate) - 14.06 \quad \text{.....(3.13)}$$

3.3.5.4 Vitreous Potassium and Calcium

When vitreous calcium was included in the same regression model as vitreous potassium, vitreous calcium was observed to be an insignificant (P, 0.57) predictor of PMI in presence of vitreous potassium. The corresponding derived multiple regression was:

$$PMI = 3.31\ (Potassium) + 3.54\ (Calcium) - 18.12 \quad \text{..... (3.14)}$$

Overall, only vitreous xanthine and lactate were the significant predictors of PMI along with the vitreous potassium in the same model. The degree of correlationship of potassium with hypoxanthine, xanthine, lactate and calcium were almost similar to potassium used alone in the model. However, a higher degree of correlationship was noted for vitreous potassium when xanthine (R, 0.765; $P< 0.0001$) and lactate (R, 0.774; $P< 0.0001$) were included in the same regression model.

3.3.6 Vitreous Biochemical Constituent Correlation with PMI - Specific Diagnostic Sub-groups

From the larger group of subjects (61 subjects) in whom an accurate PMI was known, smaller subgroups were devised based on the specific pathologic cause of death as was reported in the final autopsy record. Subsequently, smaller subgroups

of death were classified as deaths associated with cardiovascular causes, malignancies and acute trauma. In addition, one more sub group of subjects with established diagnoses of Diabetes Mellitus was classified. The purpose of this classification was to study if any additional vitreous biochemical constituent was significantly correlated with PMI to help in estimating PMI in these specific diagnostic subgroups.

3.3.6.1 Deaths Associated with Cardiovascular Disease

The linear regression correlation analyses of various vitreous biochemical constituents and PMI in this subgroup are summarized in Table. 9. Only vitreous potassium (R, 0.729; $P< 0.0001$), hypoxanthine (R, 0.625; $P< 0.01$), xanthine (R, 0.554; $P< 0.05$) and lactate (R, 0.657, $P< 0.01$) were significantly correlated with PMI in this subgroup of deaths.

3.3.6.2 Deaths Associated with Malignancies

The linear regression correlation analyses of various vitreous biochemical constituents and PMI in this subgroup are summarized in Table. 10. Only vitreous potassium (R, 0.892; $P< 0.01$) and calcium (R, 0.788, $P< 0.05$) were significantly correlated with PMI in this subgroup of deaths.

3.3.6.3 Deaths Associated with Acute Trauma

The linear regression correlation analyses of various vitreous biochemical constituents and PMI in this subgroup are summarized in Table. 11. Only vitreous potassium (R, 0.956; $P< 0.05$) was significantly correlated with PMI in this subgroup of deaths.

Table. 9. Linear regression correlation analyses of various vitreous biochemical constituents and postmortem interval in subjects dying of cardiovascular disease associated causes.

Constituent	**n**	**R**	***P* value**
Potassium	21	0.729	<0.0001
Hypoxanthine	21	0.625	<0.01
Xanthine	17	0.554	<0.05
Lactate	17	0.657	<0.01
Calcium	22	0.221	0.324
Sodium	21	0.282	0.215
Chloride	21	0.188	0.415
Magnesium	17	0.181	0.486
Urea	22	0.331	0.133
Creatinine	21	0.227	0.323
Glucose	20	0.215	0.363
Osmolality	16	0.171	0.527
Lipid hydroperoxides	19	0.017	0.943

Table. 10. Linear regression correlation analyses of various vitreous biochemical constituents and postmortem interval in subjects dying due to malignancy associated conditions.

Constituent	**n**	**R**	***P* value**
Potassium	8	0.892	<0.01
Hypoxanthine	6	0.666	0.149
Xanthine*	3	-	-
Lactate	8	0.252	0.546
Calcium	8	0.788	<0.05
Sodium	8	0.108	0.80
Chloride	8	0.433	0.284
Magnesium	8	0.416	0.305
Urea	8	0.227	0.589
Creatinine	8	0.026	0.951
Glucose	8	0.261	0.533
Osmolality	6	0.436	0.387
Lipid hydroperoxides	6	0.426	0.40

* Small ‘n’

Table. 11. Linear regression correlation analyses of various vitreous biochemical constituents and postmortem interval in subjects dying due to acute traumatic conditions.

Constituent	**n**	**R**	***P* value**
Potassium	7	0.956	<0.05
Hypoxanthine	7	0.708	0.075
Xanthine	6	0.685	0.134
Lactate	5	0.771	0.127
Calcium	7	0.463	0.296
Sodium	7	0.191	0.681
Chloride	7	0.666	0.103
Magnesium	6	0.607	0.201
Urea	7	0.653	0.112
Creatinine	7	0.253	0.584
Glucose	6	0.387	0.449
Osmolality	7	0.795	0.320
Lipid hydroperoxides	6	0.430	0.395

3.3.6.4 Subjects with an Established Diagnosis of Diabetes Mellitus

The linear regression correlation analyses of various vitreous biochemical constituents and PMI in this subgroup are summarized in Table. 12. Only vitreous potassium (R, 0.672; $P< 0.05$) and hypoxanthine (R, 0.711, $P< 0.05$) were significantly correlated with PMI in this subgroup of deaths.

In all of the classified diagnostic subgroups, vitreous potassium was a common biochemical constituent that was significantly correlated with PMI. Therefore, a statistical comparison of paired differences between actual and estimated PMI in various diagnostic subgroups was carried out using the previously derived formulae based on vitreous potassium. The results of this statistical comparison are summarized in Table. 13. The highest correlation (R, 0.956; $P< 0.05$; SD, 4.99) between actual and estimated PMI was observed in the subgroup comprising of deaths associated with acute trauma.

3.4 ANTEMORTEM SERUM AND POSTMORTEM VITREOUS BIOCHEMISTRY CORRELATION

The linear regression correlation analyses of the nine biochemical constituents for the serum values obtained within the 24 hours preceding death and the corresponding postmortem vitreous biochemical concentration is summarized in Table. 14.

3.4.1 Antemortem Serum and Postmortem Vitreous Urea

As shown in Fig. 15, the linear regression correlation analysis between antemortem serum urea and postmortem vitreous urea concentrations demonstrated a highly significant correlation (R, 0.967; $P< 0.0001$).

Table. 12. Linear regression correlation analyses of various vitreous biochemical constituents and postmortem interval in subjects with an established diagnosis of Diabetes Mellitus.

Constituent	**n**	**R**	***P* value**
Potassium	11	0.672	<0.05
Hypoxanthine	9	0.711	<0.05
Xanthine	8	0.682	0.062
Lactate	8	0.542	0.165
Calcium	11	0.025	0.941
Sodium	11	0.066	0.847
Chloride	11	0.162	0.635
Magnesium	7	0.314	0.492
Urea	10	0.049	0.886
Creatinine	10	0.024	0.949
Glucose	11	0.364	0.271
Osmolality	7	0.170	0.716
Lipid hydroperoxides	9	0.073	0.852

Table. 13. A summary of the statistical comparisons of differences between actual and estimated postmortem interval in various groups based on the derived potassium formula in the present study

Group	**n**	**R**	**Mean**	**Std. Deviation**	**Std. Error Mean**	***P* value**
All	58	0.731	0.01	14.59	1.92	<0.0001
Cardiovascular	21	0.729	3.27	16.07	3.51	<0.0001
Malignancy	8	0.892	3.13	12.68	4.48	<0.01
Diabetic	11	0.672	3.08	16.79	5.06	<0.05
Acute Trauma	7	0.956	10.38	4.99	2.23	<0.05

Table. 14. Linear regression correlation analyses of antemortem serum and postmortem vitreous biochemical constituents.

Constituent	**n**	**R**	***P* value**
Urea	25	0.967	<0.0001
Creatinine	25	0.865	<0.0001
Calcium	8	0.507	0.20
Sodium	25	0.344	0.092
Chloride	25	0.166	0.427
Magnesium	16	0.189	0.482
Potassium	24	0.186	0.385
Lactate	5	0.487	0.405
Glucose	19	0.011	0.965

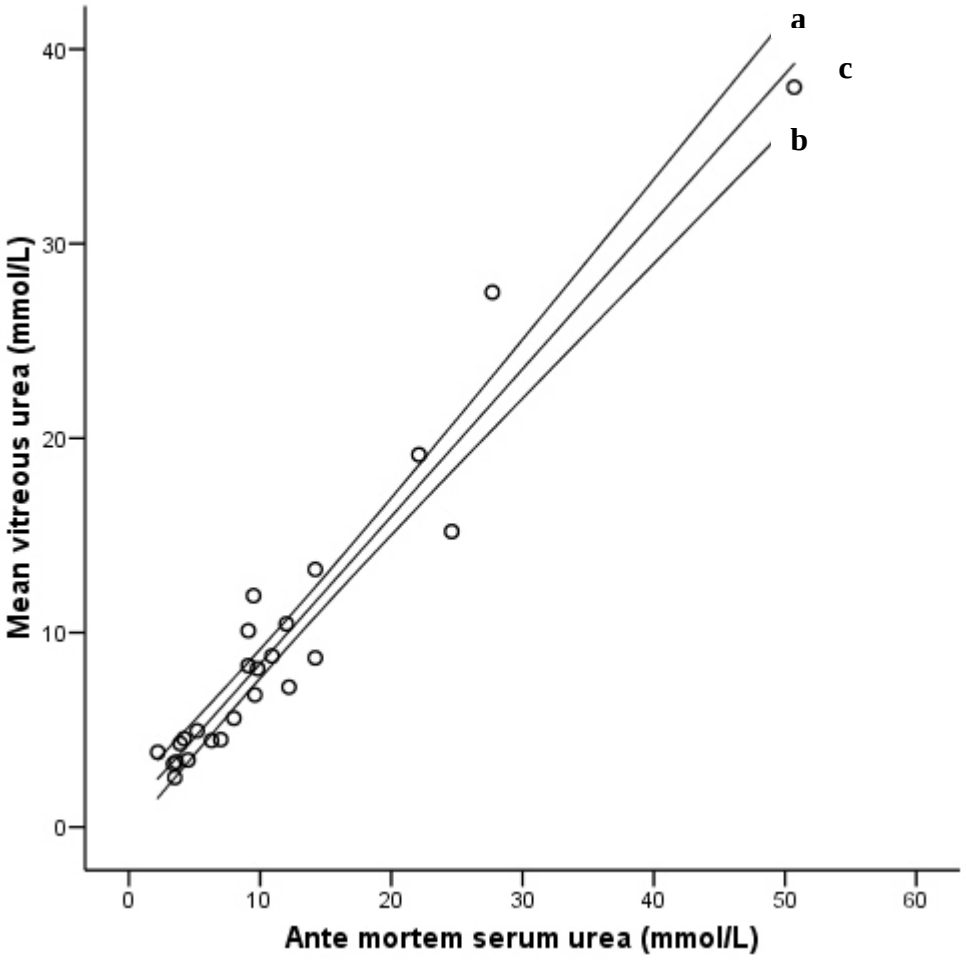

Fig. 15. Postmortem vitreous humor urea concentration compared with antemortem serum urea concentration with the 95% confidence interval bands (a and b) of the regression line (c). The graph reveals the high degree of correlation between the two measurements.

3.4.2 Antemortem Serum Creatinine and Postmortem Vitreous Creatinine

As shown in Fig. 16, the linear regression correlation analysis between antemortem serum creatinine and postmortem vitreous creatinine concentrations demonstrated a highly significant correlation (R, 0.865; $P< 0.0001$).

Apart from these two biochemical constituents, urea and creatinine, none of the other studied constituents demonstrated a significant correlationship between the antemortem serum and postmortem biochemical concentrations.

3.5 UTILITY OF VITREOUS BIOCHEMISTRY IN POSTMORTEM DIAGNOSES OF DIABETIC OR HYPERGLYCEMIC STATUS

Table. 15. shows the various statistical parameters related to differences in vitreous glucose, lactate, sum of glucose and lactate, and lipid hydroperoxides in the two diagnostic sub-groups of diabetics and non-diabetics.

3.5.1 Vitreous Humor Glucose

The mean vitreous glucose levels were found to be higher in the diabetic group and significantly different ($P< 0.05$) from the glucose levels observed in the non-diabetic subjects.

3.5.2 Vitreous Humor Lactate

There were no significant differences observed in the lactate levels of the two groups.

3.5.3 Sum of Vitreous Humor Glucose and Lactate Measurements

There were no significant differences observed in the sum of the levels of glucose and lactate of the two groups.

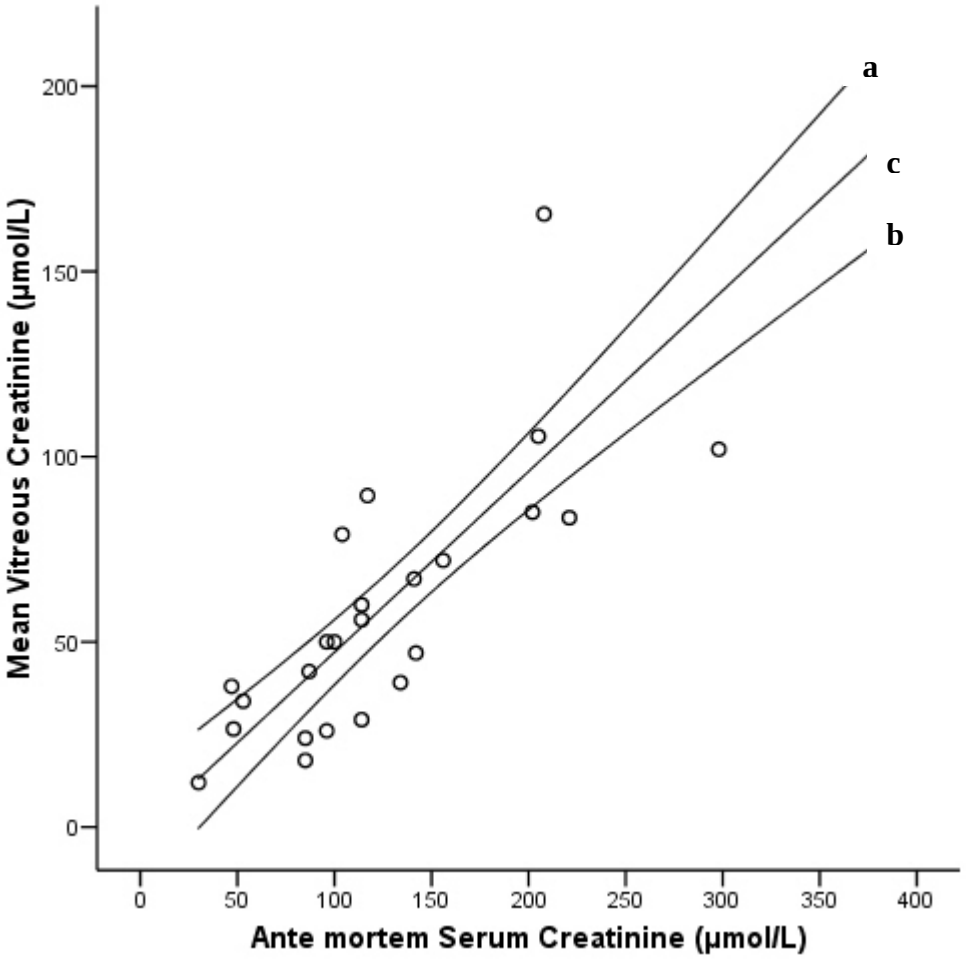

Fig. 16. Postmortem vitreous humor creatinine concentration compared with antemortem serum creatinine concentration with the 95% confidence interval bands (a and b) of the regression line (c). The graph reveals the degree of correlation between the two measurements.

Table. 15. Comparison of various vitreous humor biochemical parameters in diabetics and non-diabetics.

Constituent	**Diabetics**	**Non diabetics**	***P* value**
	Mean ± SD (n)	**Mean ± SD (n)**	
Glucose (mmol/L)	3.33 ± 3.59 (18)	1.4 ± 1.8 (54)	<0.05
Lactate (mmol/L)	9.30 ± 6.68 (14)	11.19 ± 5.75 (53)	0.291
Glucose + Lactate (mmol/L)	11.27 ± 7 (13)	11.73 ± 5.81 (42)	0.766
Lipid hydroperoxides (μmol/L)	21.78 ± 21.32 (16)	16.06 ± 10.59 (52)	0.834

3.5.4 Vitreous Humor Lipid Hydroperoxides

There were no significant differences in the lipid hydroperoxide levels of the two groups.

4.0 DISCUSSION

4.1 BETWEEN-EYE DIFFERENCES

The results of the present study suggest that the between-eye concentration differences evident in the same pair of eyes at identical PMI are not significant, and vitreous potassium levels for individual eyes, as well as mean paired concentrations, were significantly correlated with PMI. Some early studies had reported that vitreous samples obtained from the same pair of eyes had near-identical biochemical values for the two eyes (Sturner and Gantner, 1964a; Coe, 1969). These investigators, however, did not provide the data or their statistical interpretation. In the present study, we observed identical values in only a small percentage of the paired samples analyzed.

The present study findings are consistent with the conclusions of Tagliaro et al. (2001) who confirmed through a microsampling technique and capillary electrophoresis that no statistically significant differences existed for potassium concentrations in the two eyes of the same individual. The microsampling technique, aspiration of microliter amounts of fluid, used in their study is different from the technique of complete fluid aspiration employed in the present study. The findings of the present study could not confirm previous observations that suggested high between-eye differences for vitreous constituents, including potassium (Balasooriya et al. 1984; Madea et al. 1989). Although no statistical analysis of their

data was provided, these authors suggested relevant differences. Pounder et al (1998) suggested significant differences for vitreous potassium between the two eyes of the same individual. This study results do not support their conclusion about vitreous potassium but are in agreement with their findings of no significant differences in the same pair of eyes for sodium and chloride. A principal reason for the conflicting reports about the between-eye differences at identical PMI may be the variations in study methods and possible sample manipulations before analyses. An obvious discrepancy may be the aspiration techniques adopted by some investigators. Bito (1977) reported that the concentrations of many solutes in the vitreous humor are different in anterior and posterior vitreous chambers. It has also been suggested that the concentration of vitreous solutes next to the retina is different than the concentration in the central portion of the globe, and therefore it is essential to aspirate vitreous humor as completely as possible to reflect accurately the concentration levels of all solutes (Coe, 1989). This sampling technique serves to eliminate any discrepancy that may arise as a result of selective vitreous humor aspiration from regions of higher or lower solute concentrations. The aspiration technique employed by Balasooriya et al. (1984) as they aspirated only the initial 1 mL volume of fluid, could highly distort values in each eye. By contrast, results of earlier investigators (Coe, 1969) who rigorously aspirated all the available vitreous humor from both the eyes demonstrated near identical concentrations for both eyes. However, this may not be the only reason that accounts for the reported between-eye differences as supported by two previous studies (Madea et al. 1989; Pounder et al. 1998). Pounder et al. (1998) assessed the effect of the sampling technique of the

vitreous humor by aspirating the fluid in two installments and did not find any major influence of the sampling technique on the observed between-eye differences. Previously, Madea et al. (1989) strictly followed Coe's recommendation and still observed high differences in a single pair of eyes, even in the early PMI. Moreover, the microsampling technique of Tagliaro et al. (2001) also demonstrated that no significant differences exist between the same pair of eyes. Though the complete aspiration technique may be ideal to reflect accurately vitreous solute concentration levels, certain other factors may also account for the between-eye differences.

The differences in findings may also be attributed to the instrumentation methods used in different studies as it has been suggested that the concentration of vitreous humor constituents will vary with different instruments (Coe and Apple, 1985). It is also interesting to note that in studies that have suggested notable differences between the same pair of eyes, the specimens were analyzed by direct or indirect potentiometry (Balasooriya et al. 1984; Madea et al. 1989). In contrast, workers who found near-identical concentration for various vitreous humor constituents used flame photometry for their analyses (Sturner and Gantner, 1964; Coe, 1969) The biochemical analyses in the study by Pounder et al. (1998) was done by ion-specific electrode, which is a similar technique as used in the present study. Similarity in instrumentation techniques may partially explain the agreement of results for vitreous humor sodium and chloride in the two studies. Since most of the analytical instruments used in various studies have been used for a clinical range of analysis, compensatory dilution has been essential in estimating a value for most of the postmortem vitreous humor constituents. It has been hypothesized that sample

dilution prior to analysis account for the between-eye differences in the same pair of eyes, and therefore measuring the samples undiluted has been suggested (Pounder et al. 1998). In the present study, appropriate dilutions were made and our results do not suggest compensatory dilution to be critical in the biochemical analysis of vitreous constituents. Also, other studies that have reported no significant between-eye differences for vitreous constituents have also performed the required dilutions (Sturner and Gantner, 1964; Tagliaro et al. 2001).

The long time lag between vitreous-humor sample collection and analysis of the sample may be an additional factor that may explain the reported between-eye differences in the same pair of eyes. In some studies, the sample was kept frozen at -70°C before biochemical analysis (Madea et al. 1989). The inconsistent storage conditions may have influenced the results to a certain degree and it is suspected that after indefinite storage at low temperatures, results may not accurately represent the biochemical concentrations of the vitreous humor. This may be true in view of recent observations of small but significant increases in vitreous electrolyte concentrations in specimens refrigerated for 6 to 12 months (Gagajewski et al. 2004). In the present study, the suspected influence of sample storage on vitreous humor biochemical values was eliminated by immediate biochemical analyses of the samples without any prior freezing. The present study technique of immediate analysis post-collection is in accordance with the technique adopted by Pounder et al. (1998). However, in spite of a similar process, they reported significant and erratic between-eye differences for potassium. The present study methods and data are comparable with their findings for sodium and chloride but not for potassium.

The reasons for the significant between-eye differences reported for potassium in their study are still not entirely clear and in absence of any comments on paired eye correlation, comparison with their study becomes difficult. However, a possible explanation for the inconsistency could be the inclusion of cases in advanced PMI range in their study design as compared with that used in the present study.

The conflicting views in literature on the subject appear to be a result of non-uniform study methodologies and sample manipulations. The present study has attempted to eliminate most of the methodological limitations of sampling techniques and biochemical analysis evident in some previous studies. The insignificant vitreous potassium between-eye differences and a highly significant paired correlation supported by a similar linear correlation at identical levels of significance for right and left eyes with PMI suggest that vitreous potassium is a valuable biochemical marker in PMI estimation. The present study resolves the issue of between-eye differences at identical PMI for vitreous electrolytes and various other vitreous biochemical constituents. The study clearly suggests these differences to be insignificant and therefore the validity of postmortem vitreous-humor analysis in forensic pathology applications cannot be solely questioned on the basis of these differences.

4.2 VITREOUS BIOCHEMISTRY CORRELATION WITH PMI

During the course of an investigation of death, the onus is on the consulting pathologist to accurately estimate the time of death of the deceased individual. Vitreous humor is a fairly stable fluid in the postmortem period that can be utilized in death time estimations. In the present study, apart from vitreous potassium, many

other vitreous analytes have been investigated to establish their correlationship with PMI or time of death. A significant linear correlationship, at various degrees, was seen to exist between PMI and vitreous potassium, hypoxanthine, xanthine, lactate and calcium. The highest degree of correlationship was observed with vitreous potassium and PMI.

4.2.1 Vitreous Humor Potassium

In the present study, observations were made up to 84 hours (Mean ± SD, 27.9 ± 16.5) postmortem period. During the studied postmortem period, vitreous potassium represented a fairly linear rise with increasing PMI. This linear rise of vitreous potassium was consistent in the early PMI with the range of scatter increasing in the later postmortem hours especially after 50 hours into the postmortem period. These results are in accordance with previous reports in literature on the behavior of vitreous potassium in the postmortem period (Sturner, 1963; Coe, 1969; Madea et al. 1989).

4.2.1.1 Slope and Regression Equation

The slopes of the linear regression line for postmortem vitreous potassium rise against PMI reported in literature are variable and in the range of 0.14 mmol/L per hour (Sturner, 1963) to 0.332 mmol/L per hour (Coe, 1969). Similarly, the zero hour intercepts reported in literature vary from 4.2 mmol/L (James et al. 1997) to 8.0 mmol/L as reported by Hansson et al. (1966). The vitreous potassium slope reported by Sturner (1963), based on his study data corresponded with an approximate zero hour intercept of 5.6 mmol/L. Coe (1969) obtained a biphasic slope, a steep 0.332 mmol/L per hour in the initial six hours PMI and a flatter 0.16 mmol/L per hour in

the later hours with zero hour intercepts of 4.99 and 6.19 mmol/L respectively. The study by Madea et al. (1989) reported a slope of 0.19 mmol/L per hour and a zero hour '*y*' intercept of 5.88 mmol/L. James et al. (1997) from his data on vitreous potassium obtained a slope of 0.23 mmol/L per hour with a zero hour '*y*' intercept of 4.2 mmol/ L. The slope of the regression line for postmortem vitreous potassium rise with increasing PMI obtained in the present study is 0.16 mmol/L per hour with a zero hour '*y*' intercept of 7.22 mmol/L. The slope and the intercepts observed for the vitreous potassium in the present study is comparable to that reported for vitreous potassium rise in literature. The variation in experimental methods and the sample characteristics may account for the small differences noted with the slopes of various other studies. The slope of 0.16 mmol/L is steeper than that reported earlier by Sturner (1963) and slightly flatter than that reported by Madea (1989), the authors of two popular studies who have devised formulae for estimation of PMI based on these regression slopes. The regression slope for potassium obtained in the present study is in close agreement to the slope of 0.17 mmol/L per hour as obtained in a combined evaluation of the original data for potassium of six studies (Lange et al. 1994). It is essential that the slope of regression line be relatively steeper because flatter slopes tend to overestimate the time since death based on the obtained regression line and equation.

4.2.1.2 Regression Formula

Based on the regression equation obtained from the data in this study, a new formula, *PMI (hours) = 6.41 (Potassium) – 46.25*, is proposed. The advantage of this formula over some other previously proposed formulae is it's standardized

nature. The present study in deriving the formula has eliminated most of the methodological limitations that are evident in some previous studies (Sturner, 1963; Madea et al. 1989; James et al. 1997). In this study, the PMI range used in deriving the regression equation was 4.5 to 84.3 hours with a mean of approximately 30 hours. The wide PMI range used in the study has adequately accounted for the bi-phasic rise of vitreous potassium in the postmortem phase and has arrived at the ideal equation to estimate PMI over a large time period. At the same time, eliminating vitreous samples obtained from subjects with a prolonged PMI, the formula minimizes the opportunity of skewing the results with samples in late putrefaction and subsequent artefactual rise in vitreous potassium levels. Sturner (1963) proposed his formula, *PMI = 7.14 (Potassium) – 39.1*, for PMI estimation based on a similar sample size as that employed in the present study, but the relatively shorter PMI range studied limits the usefulness of his formula for wide use. In addition, the flatter regression slope obtained from his data tends to overestimate the PMI (Madea et al. 1990a; Gamero et al. 1992). Along with Sturner's formula the other formula, *PMI = 5.26 (Potassium) – 30.9*, devised by Madea et al. (1989) is widely used in forensic pathology casework. The latter formula with a desirable steeper slope and a fairly large sample size is very similar to the formula devised from the present data. However, they have not adequately addressed the large PMI range of subjects studied, up to 140 hours, and the possible influence of the putrefactive process in many of the vitreous samples. Another popular formula proposed by James et al. (1997), *PMI = 4.32 (Potassium) – 18.35*, suffers a methodological limitation as opposed to our study. James et al. (1997) did

not discard the vitreous samples, which were not crystal clear and hence may have been contaminated with adjoining tissue fragments. The use of cellular debris contaminated samples may tend to produce an artefactual increase in actual potassium concentrations of the vitreous humor. Apart from these limitations, many of the studies did not analyze the sample immediately after extraction, which itself may potentially influence the vitreous biochemical measurements (Gagajeweski et al. 2004). In the present study, rigorous sampling techniques avoiding any tissue contamination and immediate analysis of vitreous fluid post-extraction may have controlled many of the sampling variability associated with some previous studies.

Using the vitreous potassium data of the present study, a comparative testing of the present study formula with some of the previously proposed formulae by Sturner (1963), Madea et al. (1989) and James et al. (1997) was made. The deviations obtained between the estimated PMI using the present study formula and the actual PMI is represented in Fig. 17. A majority of these deviations were within ± 10 hours range and approximately 10 percent of these estimations were near identical. The deviation plot obtained from the present study formula compared very well with the deviations observed using the Madea et al. (1989) formula as seen in Fig. 18. In many of the cases, the data points between the two plots were near identical. The deviations tended to widen as the PMI period increased and the maximum deviations between the actual and estimated PMI were seen in the PMI phases greater than 48 hours. These observations are very consistent with previous reports

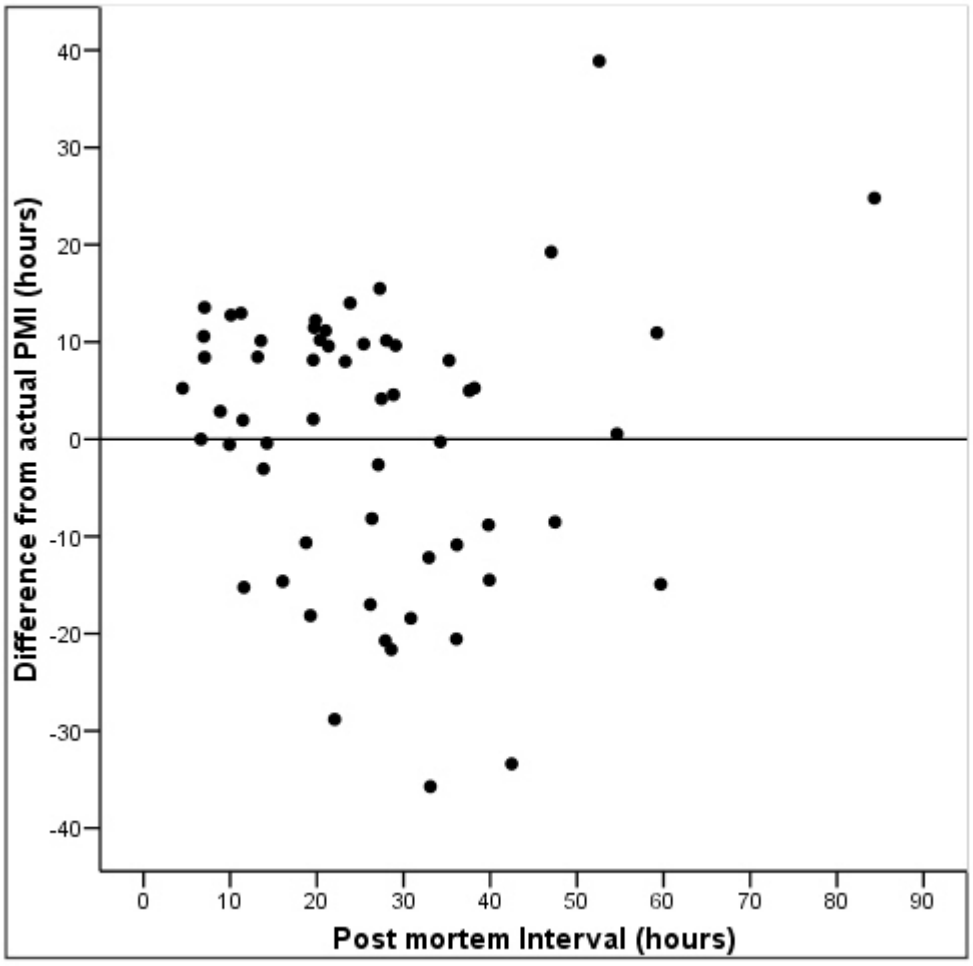

Fig. 17. Deviations between the estimated and actual PMI (hours) over the PMI studied using the vitreous humor potassium formula derived from the present study.

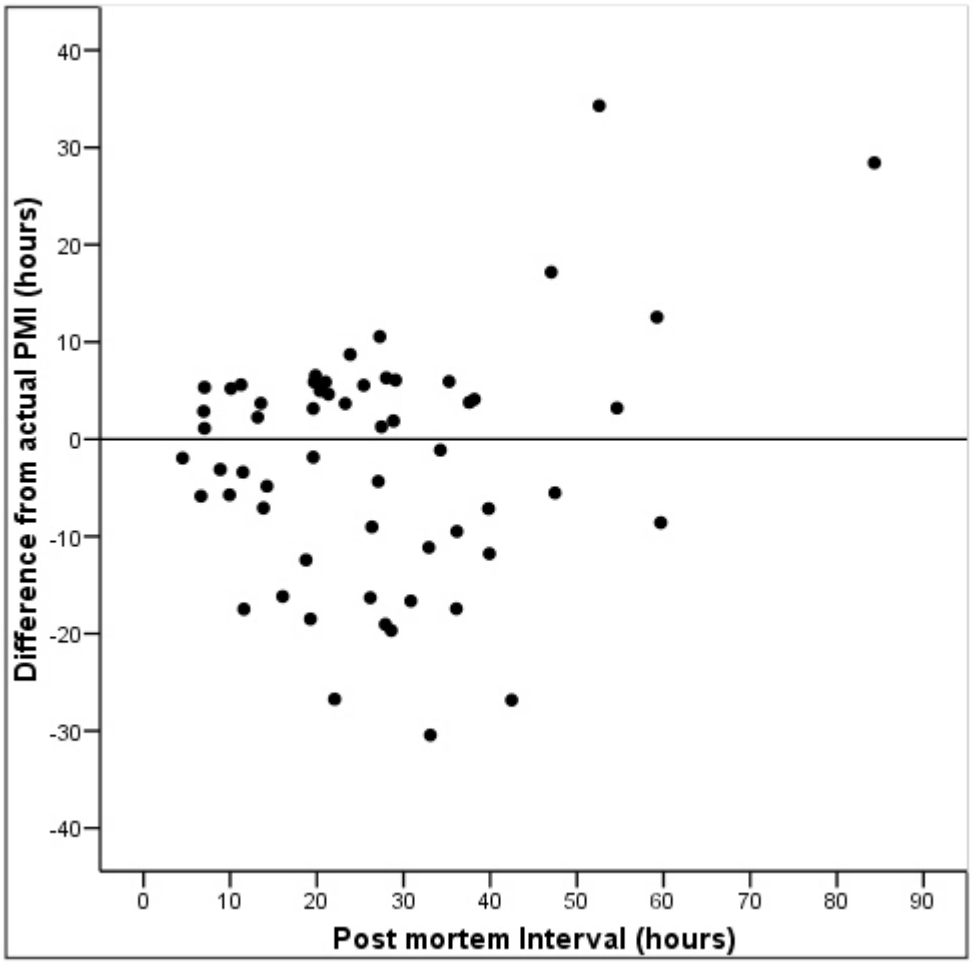

Fig. 18. Deviations between the estimated and actual PMI (hours) over the PMI studied using the vitreous humor potassium formula proposed by Madea et al. (1989).

that suggest PMI estimations using vitreous potassium levels are more reliable in the early postmortem period and the margin of error increases with increasing PMI (Coe, 1989). The deviation plot obtained by using the Sturner (1963) formula is represented in Fig. 19. It was noticed from the deviation plot that the Sturner formula with a relatively flat slope tended to systematically overestimate the PMI prediction which is consistent with previous observations (Madea et al. 1990a; Gamero et al. 1992; James et al. 1997) and raises serious questions about using this formula for future case work PMI estimations. The deviations between the actual and the estimated PMI observed using the formula proposed by James et al. (1997) is shown in Fig. 20. Overall, the standard deviations obtained by comparing the actual and estimated PMI using the different formula suggested that the deviation was the least for the formula proposed by James et al. (1997) of approximately 11.1 hours and a highest of 16.4 hours with the Sturner formula (Sturner, 1963). The standard deviation obtained on a paired comparison of actual and estimated PMI using the present study formula was approximately 14.6 hours which compared well with the standard deviation of 12.3 hours obtained using Madea et al. equation (Madea et al. 1989). The larger sample size utilized in the study by Madea et al. (1989) may have contributed to a formula that resulted in a smaller deviation as compared to that obtained using the present study formula.

4.2.2 Vitreous Humor Hypoxanthine

The results in the present study suggest a significantly correlated and linear rise of vitreous hypoxanthine with PMI. The postmortem rise of vitreous hypoxanthine was first observed in cerebrospinal fluid (CSF) (Praetorius et al., 1957). The rise in

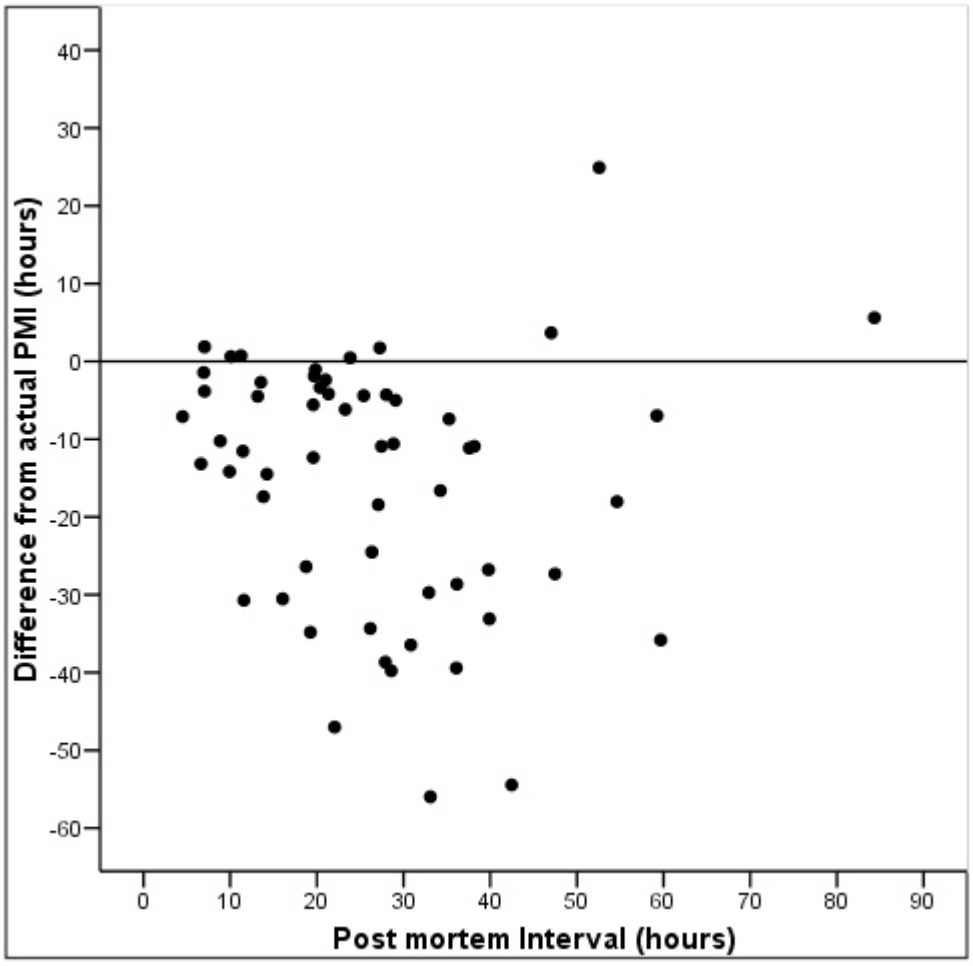

Fig. 19. Deviations between the estimated and actual PMI (hours) over the PMI studied using the vitreous humor potassium formula proposed by Sturner (1963). The systematic overestimation of PMI can be appreciated in the deviation plot.

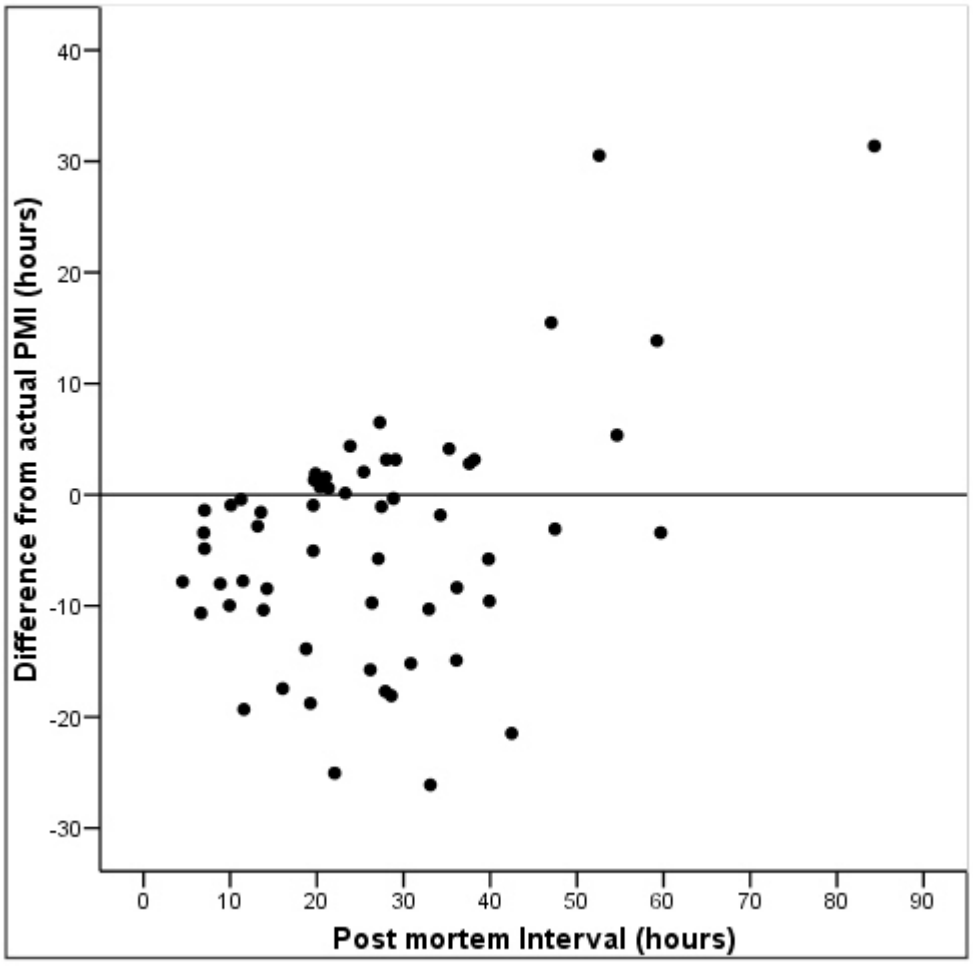

Fig. 20. Deviations between the estimated and actual PMI (hours) over the PMI studied using the vitreous humor potassium formula proposed by James et al. (1997).

postmortem vitreous hypoxanthine levels were first reported by Saugstad and Oliasen (1978). The postmortem increase in vitreous hypoxanthine levels was noted to be independent of the postmortem time during the first 48 hours and was estimated to be stable during the first 72 hours after death (Saugstad and Oliasen, 1978). In the present study, an immediate postmortem increase in vitreous hypoxanthine levels was evident and there was no stable phase of 48 or 72 hours as reported previously. The immediate postmortem rise of vitreous hypoxanthine levels is in accordance with the findings of Gardiner et al. (1989) and Rognum et al. (1991) from experimental animal and human data respectively. The postmortem vitreous hypoxanthine rise was significantly correlated with increasing PMI. However, this correlation was much stronger for vitreous potassium and PMI ($R = 0.731$) as compared to vitreous hypoxanthine levels ($R = 0.450$). In this aspect, the present study results differ from the findings of Rognum et al. (1991) who suggested a stronger correlationship between vitreous hypoxanthine and PMI as compared to vitreous potassium. Similarly, the conclusion that the range of scatter was greater for vitreous potassium than vitreous hypoxanthine as suggested by Rognum et al. (1991) could not be justified from the present data. The results from the present study, in fact, suggested that the range of scatter was greater for vitreous hypoxanthine than potassium, which is in complete agreement with the findings of Madea et al. (1994). A possible explanation for the discordant findings in the present study and the study by Rognum et al. (1991) could be due to the improper vitreous sampling methods employed by these investigators. In collecting the vitreous sample, Rognum et al. (1991) employed a multiple sample taking

methodology by which repeated aspiration of small amounts of vitreous fluid was performed from the same globe. This technique may serve to disturb the intraocular diffusion gradients and establish a drainage effect as previously described (Madea et al. 1994). In addition, the increased chances for adjoining tissue fragmentation as a result of repeated sampling and the resultant artificial increase in various vitreous solute concentrations could also explain the different conclusions in the two studies. To avoid sampling discrepancies, it is therefore essential to avoid repeated sample taking and extract the whole vitreous humor with a single aspiration of the globe. In the present study, this technique as proposed by Coe (1989) has been rigorously followed. The wider range of scatter observed for vitreous hypoxanthine as compared to vitreous potassium may also be attributed to the metabolic parameters and postmortem behavior of these two analytes. Potassium concentration in life is tightly regulated within a narrow range. In the postmortem period, after the loss of selective membrane permeability, potassium diffuses into the vitreous in a steady fashion along the concentration gradient from the retina to the centre of the vitreous. Hypoxanthine, as described before, is a vital degradation product of purine metabolism, which is a marker of tissue hypoxia. It increases in the postmortem period and mainly diffuses from the retina into the centre of the vitreous. It is therefore to be expected that an analyte that follows steady diffusion in the postmortem period will more reliably predict PMI than hypoxanthine, which increases mainly due to postmortem degradation (Madea et al. 1994).

The slope obtained for the vitreous hypoxanthine rise in the postmortem period from the present study was 3.2 μmol/L per hour. This slope is identical to the

slope reported in the study by James et al. (1997) and comparable to the slope of 3.01 μmol/L per hour as reported by Munoz et al. (2002). On a paired comparison of estimated PMI using the hypoxanthine-based formula and the actual PMI a statistically significant correlation was noted with a standard deviation of 32.55 hours. Similarly, substituting the hypoxanthine values in the earlier reported formulae (James et al. 1997; Munoz et al. 2002), the observed standard deviations were 31.23 hours and 19.33 hours respectively. These deviations are similar to that obtained using the present study hypoxanthine-based formula. Different measurement techniques of vitreous hypoxanthine may account for the small differences noted in the results from the present study and the results of the previous studies. A relatively sensitive method of High Performance Liquid Chromatography (HPLC) was employed in the earlier two studies as compared to the colorimetric commercial kit utilized for vitreous hypoxanthine measurements in the present study. The colorimetric commercial kit used in the present study offers a simple and cost effective methodology with comparable results to the HPLC method in vitreous hypoxanthine measurements.

4.2.3 Vitreous Humor Xanthine

Similar to hypoxanthine, xanthine is an oxypurine and a metabolic product obtained in the last stages of purine catabolism in humans. Due to similarities in the metabolic pathways of hypoxanthine and xanthine, the present study evaluated the usefulness of measuring vitreous xanthine levels in estimation of PMI. The results of the present study suggest that vitreous xanthine increases in a fairly linear manner in the postmortem period. This increase in vitreous xanthine levels is significantly

correlated (R = 0.590; P<0.0001) to PMI. There are few published works with which a comparison of postmortem vitreous xanthine levels can be made. The rise of vitreous xanthine in the postmortem period is consistent with the earlier reports of postmortem increase of xanthine levels in postmortem chicken and porcine vitreous humor (Gardiner et al. 1989; Stoltenberg et al. 1993). Although, the possibility of species difference must be adequately recognized, the present study data indicates a similar level of significance (P< 0.0001) and rise in postmortem vitreous xanthine concentrations as reported for chicken (Gardiner et al. 1989). The present study is the first one to derive a linear regression equation for the postmortem vitreous xanthine rise in humans and to establish a formula based on the regression equation. After substituting the vitreous xanthine values in the proposed formula and comparing the estimated PMI with the actual PMI, a highly significant correlation was obtained between the two groups with a standard deviation of 20.40 hours, which was smaller than the standard deviation obtained with vitreous hypoxanthine. At the same time, the correlation of vitreous xanthine and PMI was higher than that observed with vitreous hypoxanthine and PMI. In light of the present findings, a re-analysis of vitreous xanthine as a better indicator of PMI than vitreous hypoxanthine is warranted.

4.2.4 Vitreous Humor Magnesium and Calcium

The vitreous calcium and magnesium concentrations observed in the present study are very similar to the concentrations previously reported (Coe, 1969; Nowak and Balabanova, 1989). There was a significant correlation observed for vitreous calcium and PMI (R = 0.33; P < 0.01) but not vitreous magnesium and PMI. The

significant correlation of vitreous calcium and PMI observed in the present study is similar to the findings of Nowak and Balabanova (1989). However, in the study by Nowak and Balabanova, the significant correlation between vitreous calcium and PMI was only noted in specific groups of death comprising of heart disease and asphyxia. Although, in the present study, a significant correlation was observed between vitreous calcium and PMI in the total samples studied and was not restricted to certain groups of deaths. On a paired comparison using the calcium-based formula between the estimated PMI and actual PMI, a standard deviation of 46. 80 hours was observed. When the total group of deaths were further sub classified based on the particular autopsy diagnoses, the significance was restricted to deaths associated with complications due to malignancies ($n = 8$; $R = 0.788$, $P < 0.05$). The present study does not support the previous finding of a significant correlation between postmortem vitreous calcium and PMI in deaths associated with cardiovascular disease (Nowak and Balabanova, 1989). The small number of subjects with cardiovascular disease associated deaths in the present study and variable sample characteristics may be partly responsible for the different results in the two studies. In addition, an almost horizontal slope of 0.01 mmol/L per hour noted in the present study and a wide scatter of postmortem calcium values make the determination of PMI based on calcium levels difficult and less reliable. The relative stability of postmortem vitreous calcium levels has been previously reported to be a useful indicator of antemortem calcium balance (Choo-Kang et al. 1983). Madea et al. (1990b) also reported a similar significant correlationship ($R = 0.356$) between postmortem vitreous calcium and PMI but disagreed on the utility of using

vitreous calcium in PMI prediction because of a near horizontal slope and range of scatter.

The present study did not find a significant correlationship between vitreous magnesium and PMI. The present study results are in variance with the earlier reported utility of vitreous magnesium in PMI predictions (Nowak and Balabanova, 1989; Balabonava and Gras, 1992). However, these reports were based on data in only particular groups of death like asphyxia and deaths due to phenobarbital intoxications. In the present study, useful correlation between PMI and vitreous magnesium could not be observed in the total samples or in the various diagnostic sub-groups studied. Our results are consistent with the earlier findings that reported no significant correlation between vitreous magnesium and PMI (Gregora et al. 1979; Wheeler et al. 1983; Farmer et al. 1985). The relatively small sample size in the various diagnostic sub-groups may limit the utility of the present study results with regards to usefulness of vitreous calcium and magnesium in particular sub-groups of death.

4.2.5 Vitreous Humor Lactate

Vitreous lactate exhibited a significant correlation ($R = 0.508$; $P < 0.0001$) with PMI. The regression slope obtained with vitreous lactate and PMI of 0.19 mmol/L per hour was almost similar to the slope obtained for the vitreous potassium regression. However, the scatter for vitreous lactate values was wider and therefore less reliable than vitreous potassium. On substitution of the vitreous lactate data in the derived lactate based formula for PMI estimation from the present study, the standard deviation of paired differences between estimated and actual PMI was

27.99 hours. The standard deviation obtained in this manner was smaller than that obtained with vitreous hypoxanthine and calcium estimations but larger than potassium and xanthine estimations. In the postmortem period, glucose in the cadaver fluids is converted to lactate because of the prevalent anaerobic conditions in the cadaver. The present study confirms the linear rise of lactate in the postmortem period (Jaffe, 1962). This rise was found to be more rapid during the initial 24 to 30 hours postmortem and thereafter the rise in vitreous lactate levels was slower. Consistent with the previous findings regarding vitreous lactate, the present study confirms the highly variable changes in the vitreous lactate as compared to vitreous potassium limiting its usefulness in PMI estimations. The lesser variability and the steady rise of vitreous lactate in the initial postmortem period may make vitreous lactate based PMI estimations more reliable in the initial postmortem period.

4.3 ANTEMORTEM SERUM AND POSTMORTEM VITREOUS BIOCHEMISTRY CORRELATION

The knowledge of antemortem metabolic status of a deceased individual provides a window towards establishing the clinical condition of the deceased prior to death. In many instances the results of antemortem serum biochemistry are not available and postmortem serum biochemistry, which is subjective to postmortem contamination and degradation, may not be entirely reliable. Vitreous humor is a stable postmortem fluid and may be useful in predicting the antemortem biochemistry or the metabolic status of a deceased individual (Lane and Lincoln, 1985).

In the present study, we explored the correlation between the antemortem serum and postmortem vitreous biochemical concentrations for sodium, potassium, chloride, calcium, magnesium, glucose, lactate, urea and creatinine. The results indicated that postmortem vitreous urea (R = 0.967; $P< 0.0001$) and creatinine (R = 0.865; $P< 0.0001$) levels were highly correlated with antemortem serum levels. This finding is consistent with a few earlier observations that reported a marked stability of postmortem urea and creatinine concentrations in the vitreous humor (Wilkie and Bellany, 1982; Gregora, 1984; McLaughlin and Mc Laughlin, 1988; Hanna et al. 1990). In the postmortem period, urea concentrations in the vitreous humor remain relatively more constant as compared to serum or CSF (Nauman, 1959). Creatinine in the post mortem period remains relatively constant in both CSF and vitreous humor (Nauman, 1959). The postmortem stability of vitreous urea and creatinine and their strong correlation with the antemortem serum biochemistry is helpful in providing reliable information about the antemortem renal status of the deceased subject or in making a postmortem diagnoses of renal failure.

The postmortem stability of vitreous calcium and its correlation with antemortem calcium levels has been controversial. Some investigators have suggested that postmortem vitreous calcium concentrations are stable in the postmortem period (Coe, 1969; Blumenfeld et al. 1979; Dufour, 1982) while a few others have disagreed on these conclusions (Swift et al. 1974). This variability in findings raises many questions on using postmortem vitreous calcium concentrations in predicting the antemortem calcium status of the deceased subject. In the present study, we observed a significant increase of vitreous calcium levels in

the postmortem period and correspondingly observed that there was no significant correlation between antemortem and postmortem calcium concentrations. The slope of postmortem vitreous calcium rise against increasing PMI was found to be nearly horizontal (0.01 mmol/L per hour). In accordance with the near horizontal slope, the degree of linear correlation (R = 0.333; $P< 0.01$) observed for vitreous calcium and PMI was the lowest of all the vitreous constituents exhibiting a significant correlation with PMI. The near horizontal slope of postmortem vitreous calcium is consistent with the earlier reported findings in another autopsy-based study involving human subjects (Dufour, 1982). The results of the present study support the observation that the poor correlation between the antemortem serum and postmortem vitreous calcium concentration precludes the use of vitreous calcium in evaluating the antemortem calcium balance of an individual (Coe, 1969; Dufour, 1982). This can be explained due to the different concentration gradients that exist for calcium in a typical mammalian eye as proposed by Bito (1970). Bito, through extensive studies, observed intraocular concentration gradients for calcium, which existed even between the posterior and the anterior vitreous chambers. Bito concluded that calcium concentration in the vitreous humor is regulated by an active transport mechanism. Therefore, by virtue of this active transport mechanism, hypocalcemia may not necessarily result in low vitreous calcium levels or saturation of the transport systems may itself limit the rise in vitreous calcium levels in cases of hypercalcemia. However, it should also be stressed that in the present study, only eight comparisons between antemortem and postmortem calcium measurements were possible. The small sample size necessitates further studies with a larger

sample size to strongly establish the precise correlation between antemortem serum and postmortem vitreous calcium concentrations.

Magnesium deficiency is a common finding in alcoholics (Flink, 1986). Hypomagnesemia has also been implicated as a primary pathogenetic factor in sudden deaths due to cardiac arrhythmias, particularly alcoholics (Fisher and Abrams, 1977). Postmortem documentation of magnesium deficiency is difficult. The postmortem serum magnesium concentrations have been reported to increase in an erratic fashion due to altered membrane permeability and cell destruction. Therefore correlation of antemortem and postmortem serum concentrations is impossible. The importance of evaluating the antemortem magnesium status in establishing a postmortem diagnoses and a useful role for vitreous magnesium in diagnosing magnesium imbalances has been previously reported (Lincoln and Lane, 1985). The present study yielded a poor and insignificant correlation (R, 0.189) between antemortem serum and postmortem vitreous magnesium concentrations. The findings in the present study differ significantly from the conclusions of Lincoln and Lane (1985) through studies with healthy cattle. The difference in species or the relatively small sample size of sixteen comparisons employed in the present study may be responsible for the different findings in the two studies.

Overall, only postmortem vitreous urea and creatinine were significantly correlated with their corresponding antemortem serum concentrations. The feasibility of utilizing other vitreous biochemical constituents in predicting the antemortem biochemical status appears to be very limited and unreliable.

4.4 UTILITY OF VITREOUS BIOCHEMISTRY IN POSTMORTEM DIAGNOSES OF DIABETIC OR HYPERGLYCEMIC STATUS

Diabetes Mellitus is a chronic metabolic illness and accounts for a large number of deaths with no obvious autopsy findings or a previous history of diabetes. In such a scenario, the measurement of postmortem biochemical markers is central to providing information on the cause of death. Coe (1993) suggested that an integrated approach utilizing biochemical determinations in blood, CSF, vitreous humor and other fluids could help in solving a majority of forensic problems faced by the examining pathologist. Since glucose levels in the body after death fall rapidly due to anaerobic degradation or glycolysis (Bray et al. 1983), interpretation of postmortem glucose levels presents with unique challenges.

4.4.1 Vitreous Humor Glucose

Although a diagnosis of hypoglycemia cannot be reliably made in the postmortem period, high level of vitreous glucose levels can be considered to accurately reflect antemortem hyperglycemic status (Schoning and Strafuss, 1980). In the present study, vitreous glucose levels in the diabetic subjects were observed to be within normal clinical limits. This finding was expected since a majority of the subjects studied were derived from the hospitalized population in whom the glucose levels were adequately monitored and well controlled. However, there still existed a significant difference ($P< 0.05$) between the mean glucose levels of the diabetic and the non-diabetic subjects (Mean ± SD, 3.33 ± 3.59 vs 1.4 ± 1.8 mmol/L). It is possible that the diabetics retain higher glucose levels in the body and the rate of fall in their glucose levels may be more gradual as compared to the non-diabetics.

4.4.2 Vitreous Humor Lactate

Lactate is obtained from the anaerobic metabolism of glucose. In the postmortem cadaver fluids, glucose is converted into lactate and the lactate levels increase during the first 24 hours following death. The rise of vitreous lactate slows down in the later period after 24 hours PMI as observed in the present study. In the present study, mean vitreous lactate levels were observed to be slightly higher in the non-diabetic subjects as compared to the diabetic subjects (Mean ± SD, 11.19 ± 5.75 vs 9.30 ± 6.68 mmol/ L). These differences in the two groups were not found to be significant. These findings are different from the findings reported by Osuna et al. (2001) who reported significant differences for lactate levels between the diabetic and non-diabetic subjects. The discordant results may be a result of the small number of diabetic subjects compared with the non-diabetic subjects in the present study. The ratio of diabetics to non-diabetics utilized in the study by Osuna et al. (2001) was 1:1, whereas in the present study the ratio between diabetics and non-diabetics was approximately 1:4. It is also possible that the different modes of death and influence of agonal changes on the lactate levels could have possibly caused these discordant findings.

4.4.3 Sum of Vitreous Humor Glucose and Lactate Measurements

In order to compensate for the postmortem conversion of glucose to lactate in the vitreous humor, a combined estimation of glucose and lactate values in the two diagnostic subgroups was undertaken. The mean sum of glucose and lactate values observed in the diabetic and non-diabetic subjects was nearly identical (Mean ± SD,11.27 ± 7 vs 11.73 ± 5.81 mmol/L). No significant differences were noted in the

sum values of lactate and glucose between the two groups. These results are different from a few earlier studies (Sippel and Mottonen, 1982; Peclet et al. 1994; Osuna et al. 2001). A major reason as outlined earlier for the discrepancy in the findings of the present study and the previous studies could be the mismatched sample size of diabetic and non-diabetic control subjects.

4.4.4 Vitreous Humor Lipid Hydroperoxides

The pathogenesis of diabetes mellitus is associated with increased lipid peroxidation that may contribute towards long-term sequelae of tissue damage. In this study, we measured the lipid hydroperoxide (ROOH) content of the vitreous humor and assessed its utility as a forensic biochemical marker in the postmortem evaluation of the diabetic status. For this purpose, vitreous humor ROOH were measured using the FOX 2 assay (Nourooz-Zadeh et al. 1994) which has been previously used in establishing the differences between plasma ROOH values in diabetics and non-diabetic subjects (Nourooz-Zadeh et al. 1994, 1997; Santini et al. 1997). The results from the present study indicated that the vitreous humor ROOH levels in the diabetic subjects were higher than that measured in the non-diabetic subjects (Mean ± SD, 21.78 ± 21.32 vs 16.06 ± 10.59 μmol/L). There were no significant differences observed in the vitreous ROOH levels of the two diagnostic sub-groups.

There are no previous studies that have measured and assessed the vitreous humor ROOH in diabetic and non-diabetic subjects using the FOX 2 assay. The present study suggests that the vitreous humor ROOH is measurable using the FOX 2 assay. The results of the present study does not support some previous studies that suggested a significant increased plasma ROOH levels in the diabetics compared to

the controls (Nourooz-Zadeh et al. 1994, 1997; Santini et al. 1997). The mean plasma ROOH levels previously suggested for Type 1 (IDDM) and Type 2 (NIDDM) diabetic subjects (Santini et al. 1997; Nourooz-Zadeh et al. 1997) were lower than that measured for postmortem vitreous ROOH (Mean ± SD, 7.23 ± 2.11 and 9.4 ± 3.3 vs 21.78 ± 21.32) in the present study. Differences in the matrix may be a major reason for the inconsistent findings in the plasma and vitreous ROOH levels. The eye is a rather unique organ, relatively unprotected and constantly exposed to the environmental insults in the form of atmospheric oxygen, radiation and various chemicals. Each of these factors is related to the generation of reactive oxygen species or various free radicals, which can contribute to increased lipid peroxidation products and resulting eye damage. In contrast, plasma is relatively better protected and may escape many of the direct environmental insults unlike the eye. Therefore, it is possible that the measured vitreous humor ROOH levels in the non-diabetics may actually represent an artefactual rise as a consequence of the external environmental insults. The present study has measured the ROOH levels from a postmortem fluid obtained from the cadaver and therefore the influence of agonal changes on vitreous humor ROOH levels should also be carefully considered. Increased lipid peroxidation has also been recognized to be an important pathogenetic factor in cataract formation (Babizhayev, 1989; Babizhayev et al. 2004). It is also possible that ocular pathologies by itself may play a critical role in determining the vitreous ROOH levels and may explain the discordant findings regarding vitreous and plasma ROOH concentrations. Further studies with a larger sample size and well controlled for subject characteristics including ocular diseases

are warranted to accurately establish the value of using vitreous ROOH as possible indicators of postmortem diabetic status of an individual.

5.0 CONCLUSIONS

The principal objectives of the present study were to investigate the between-eye differences for various vitreous biochemical constituents and to evaluate the utility of vitreous humor biochemistry in making important forensic pathology determinations. The experimental results of the present study lead to the following conclusions:

1. No significant differences exist for the various biochemical constituents studied in the same pair of eyes at identical PMI.
2. The present study re-establishes and confirms a significant role for vitreous potassium in estimating PMI. The 95% confidence interval limits for the PMI estimation based on the proposed formula lies between ± 3.8 hours.
3. The other vitreous biochemical markers like hypoxanthine, xanthine, lactate and calcium, identified in the present study, can be useful adjuncts to vitreous potassium in PMI estimations.
4. Postmortem vitreous urea and creatinine concentrations are significantly correlated with the antemortem serum levels and may be helpful in establishing the antemortem metabolic status of the deceased individual.
5. Postmortem vitreous glucose levels were observed to be significantly different in the diabetic and non-diabetic subjects. The measurement of

postmortem vitreous glucose levels may aid in a postmortem diagnosis of diabetes mellitus or hyperglycemia.

The findings of this study support a central role for vitreous humor biochemistry in many postmortem forensic and pathological evaluations. The study substantially resolves the issue of significant between-eye differences for various forensically relevant vitreous biochemical constituents.

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7.0 APPENDIX

Horizontal section through the eyeball at the level of the optic nerve with the optic axis and the axis of the eye ball included. (Illustration reproduced from Clinical Anatomy of the Eye, 2nd ed, Blackwell Science Inc., with permission).