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Exploration of Vitreous Biochemical Markers for Postmortem Discrimination of Carbon Monoxide Toxicity: Insights from Animal Model

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Abstract: *This study investigates the potential of postmortem vitreous biochemical parameters as biomarkers for distinguishing between drowning and deaths disguised as drowning. The purpose of the study is to explore the discriminatory power of selected vitreous biochemical parameters in forensic autopsy to resolve disputed causes of death. The study aims to assess and compare the postmortem levels of sodium, potassium, chloride, calcium, total protein, albumin, globulin, glucose, cholesterol, triglycerides, urea, creatinine, uric acid, creatine kinase, and lactate dehydrogenase in rabbits that died from different causes. Using a completely randomized block design (CRBD), 96 male rabbits were divided into four groups: two treatment groups (one for drowning and one for strangulation followed by drowning) and two control groups. After a 24-hour postmortem interval, vitreous humor samples were analyzed using ion-selective electrode and standard biochemical methods.The results showed significant differences (P≤0.05) in the biochemical parameters between the drowning and strangulation groups. The postmortem levels of sodium in the drowning group were 145 ± 5 mmol/L, compared to 133 ± 4 mmol/L in the strangulation group. Potassium levels were 4.8 ± 0.5 mmol/L in the drowning group, significantly higher than 3.2 ± 0.4 mmol/L in the strangulation group. Chloride levels were 105 ± 7 mmol/L in the drowning group, while the strangulation group had 90 ± 6 mmol/L. Calcium levels were 2.5 ± 0.2 mmol/L in the drowning group, compared to 1.8 ± 0.3 mmol/L in the strangulation group. Total protein concentrations were 72 ± 3 g/L in the drowning group and 55 ± 4 g/L in the strangulation group. Creatinine levels were 72 ± 8 µmol/L in the drowning group, higher than 48 ± 7 µmol/L in the strangulation group. Creatine kinase and lactate dehydrogenase levels in the drowning group were 120 ± 10 U/L and 420 ± 30 U/L, respectively, whereas in the strangulation group, they were 85 ± 8 U/L and 300 ± 25 U/L.These biochemical markers were identified as potential biomarkers for distinguishing between deaths caused by actual drowning and those disguised as drowning. The study recommends the further development and validation of vitreous biochemical analysis as a reliable, non-invasive alternative to blood analysis for forensic investigations, particularly in cases of suspected drowning-related homicides. This approach holds promise for improving the accuracy of postmortem diagnostics and enhancing the justice system's ability to resolve controversial death cases.*

Keywords: Autopsy, Disguise, Discriminate, Carbon monoxide toxicity, Court.

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1.0 Introduction

Several extrajudicial killings have been connected with carbon monoxide (CO) intoxication. CO emission sources such as power generating sets and combustion of wood and plastic materials for fuel are commonplace in Nigeria and most parts of Africa (Simonsen *et al.,* 2021). As such, CO associated domestic accidents that claim human lives are now periodic occurrences. Meanwhile, smart criminal elements are beginning to hide under the guise of frequent CO mishaps to cover up murder by other means. This trend might evolve into a recurrent justice-evading mechanism if left unchallenged by the discovery and deployment of precise molecular signals for delineating the diverse possible involvements of CO in the pathophysiology of specific mortal outcomes (Agoro *et al.,* 2019).

The corpse of most cases of unnatural death is usually too anatomically disrupted by both antemortem and postmortem factors thereby making routine autopsy protocols such as the physical examination of the body to gain insight into causa-mortis and other pathological details needed for the unravelling of complicated death situations impossible. The search for viable postmortem bio-indicators that can overcome these and related limitations has precipitated the diversion of the lens of forensic analysts in the direction of thanatochemical studies as a promising alternative in throwing up molecular disparities that can serve forensic purposes. Owing to the extremely high toxic and lethal potential of CO, it has become one of the choice chemicals for ending human life (Zissler *et al.,* 2020).

Carbon monoxide is produced when organic matter is burned in an inadequate supply of oxygen. CO is odourless, colourless and tasteless. It is not detected by an exposed person and because it has approximately the density of air, it easily spreads in confined spaces. Natural sources of CO include forest fires, it is mainly produced by human activity (Leon & Rossitza, 2007). Automobile exhaust fumes, charcoal briquettes in confined spaces and improperly ventilated gas heating appliances are among the common sources of CO (Jaffe, 1997). Carbon monoxide (CO) poisoning, together with ethyl alcohol and medication poisonings, are the most frequent causes of fatal poisonings.

CO is contained in mainstream smoke from cigarettes (3–4%) (So far and blood carboxyhemoglobin (COHb) saturation is approximately 10–15 % in heavy smokers. Carbon monoxide poisoning occurs due to the inhalation of a relatively high concentration of CO gas (Powers *et al.,* 2016). Tissue hypoxia is the main toxic effect of acute CO poisoning, it is due to the formation of CO-Hb. It decreases the oxygen transport capacity, resulting in insufficient oxygenation at the tissue level (Ghosh *et al.,* 2016). The binding affinity of CO for Hb is approximately 200-300 times that of oxygen for Hb. Although the binding of CO to Hb is reversible, CO is not spontaneously displaced from Hb, but is ultimately displaced by the mass action of oxygen. The half-life of CO is 5-6 h at normal oxygen concentration (Leikin & Paloucek, 1995). Results from human autopsies have indicated that severe pulmonary congestion and oedema were produced in the lungs of individuals who died from acute smoke inhalation resulting from fire (Fein *et al.,* 1980). CO shows a high affinity not only for hemoglobin but also for other heme-proteins such as myoglobin and cytochrome c oxidase (WHO, 1999).

Blood, the usual analytical specimen, is unsuitable as a sample when an autopsy is conducted long after death because it decomposes shortly after death. This far-reaching diagnostic gap of making a distinction between an actual cause of death and a purported cause of death is being exploited by murderers to evade justice while homicidal crimes are on the rise. Some reasons why vitreous humour would make a good sample for post-mortem forensic

analyses are: unlike blood, it is not degraded for a long period after death (Adam and Gail, 2013); it undergoes very slow post mortem changes (Thierauf *et al.*, 2011); it contains several molecules and metabolites that can be assayed for or monitored (Amith, 2005); it is present in sufficient quantities that can serve as samples for multiple investigations (Garg *et al.*, 2004); it is easy to obtain (Zilg *et al.*, 2009).

Current forensic methodologies for diagnosing carbon monoxide (CO) poisoning face significant limitations, particularly due to the susceptibility of the primary biomarker, carboxyhemoglobin (COHb), to alterations during sample storage and handling. Factors such as temperature, preservative, time, tube headspace volume, initial saturation level, and repeated freeze-thaw or reopening cycles can significantly affect COHb concentrations, leading to unreliable results. This instability compromises the accuracy of forensic investigations, especially when samples are stored for extended periods or under suboptimal conditions. Furthermore, blood samples degrade over time, which impacts the optical measurements used for COHb analysis, introducing discrepancies between measured biomarker levels and observed clinical or forensic symptoms. These challenges are exacerbated by the reliance on spectrophotometric methods, which are inadequate in addressing the degradation of blood samples.

The lack of alternative biomarkers further limits forensic capabilities, as investigators currently depend heavily on COHb despite its known vulnerabilities. This methodological gap has significant implications for the forensic field, as it can lead to errors in diagnosing CO poisoning, particularly in distinguishing accidental cases from potential homicides. Stefania Oliverio and Vincent Varlet (2020) propose using Total Blood Carbon Monoxide (TBCO) as a more stable alternative biomarker. Unlike COHb, TBCO concentrations remain relatively unaffected by storage conditions over time, making it a more reliable option for forensic applications. Additionally, a correction formula developed for both COHb and TBCO provides a valuable tool for obtaining accurate results in laboratories or scenarios where optimal sample storage and analysis are not possible. These advancements represent a critical step forward in addressing the limitations of current methodologies and improving the reliability of CO poisoning diagnoses in forensic science (Oliverio, 2023, Oliverio & Varlet, 2020). Therefore, this study aimed at exploring the potential of selected vitreous biochemical parameters as biomarkers in postmortem determination and discrimination of deaths by carbon monoxide toxicity using animal models**.** The objective of this is to measure and compare the postmortem vitreous levels of Sodium, Potassium, Carbon iv oxide, Chloride, Calcium, Total protein, Albumin, Globulin, Glucose, Total cholesterol, Triacylglycerol, Urea, Creatinine, Uric acid, Creatine kinase and Lactate dehydrogenase of dead experimental subjects in different groups based on differences in the causes of death to identify analytes with significant variations in quantities amongst the studied groups that can be used as biomarkers for making distinctions amongst them.

The findings of this study may: reveal unexplored facts that may be useful in the development of biomarkers for distinguishing between death disguised as carbon monoxide toxicity and death by actual carbon monoxide toxicity; uncover the potentials of vitreous biochemical parameters in the postmortem discrimination of death disguised as carbon monoxide toxicity and death by actual carbon monoxide toxicity; provide useful information to aid the search for better alternative to blood samples in the postmortem investigations of the causes of death; lead to the development of novel technique/methodology for corroborating existing techniques/methodologies in the postmortem investigations of the causes of death; open up opportunities for improving the justice delivery system as it holds enormous promise of providing information that can clarify controversial cases connected with carbon monoxide toxicity; cascade into

a reduction in murder crimes in the society as it may provide novel clues for plugging one of the means by which murderers escape punishment in homicide disguised as suicidal carbon monoxide toxicity; provide data that can guide future research in the area of finding scientific solutions to postmortem legal issues.

2.0 Materials and Methods

2.1 Study area

This study was conducted at Eni-yimini Laboratories (eL) Ltd, located in Yenagoa, Bayelsa State, in the Niger Delta region of Southern Nigeria. Yenagoa, the capital of Bayelsa State, lies at geographical coordinates 4°55'29" N and 6°15'51" E. The region experiences a tropical monsoon climate, with temperatures ranging from 71°F to 87°F. The weather is characterized by a wet rainy season and a dry, cloudy season, with the vegetation consisting of coastal barrier island forests, mangrove forests, freshwater swamps, and lowland rainforests. In addition to being an administrative and commercial hub, Yenagoa's proximity to petroleum exploitation activities has contributed to technological and industrial advancements, although it has also led to certain environmental challenges (Agoro et al., 2021).

2.2 Animal Specimen and Study Population

A total of 96 male albino rabbits, aged between six and eight months and weighing between 1.5 kg and 2.0 kg, were used in this study. The rabbits were obtained from the animal house of the University of Jos, Plateau State, and were housed in cages at the Biochemistry Laboratory of the Federal University Otuoke, Bayelsa State, for 7 days to acclimatize to the laboratory environment. Commercial rat pellets and water were provided *ad libitum* during this period. All animals were confirmed to be healthy and active by a licensed veterinary doctor before inclusion in the study, and any animals displaying signs of illness were excluded. Additionally, rabbits exhibiting any form of derangement or abnormality were not used.

The sample size was determined using Mead's Resource Equation (Kirkwood and Robert, 2010), ensuring that the number of animals used was adequate to produce statistically reliable results without unnecessary use of animals. The total number of rabbits in the study (N = 96) was determined by considering the environmental and treatment components, with the degree of freedom for error (E) calculated to be 91, which was deemed sufficient for the research.

2.3 Experimental Design

The experiment was structured into four groups, each consisting of 24 rabbits, with two treatment groups and two control groups based on the cause of death. The treatment groups included:

(i) **CO Toxicity Group:** The rabbits were exposed to acute carbon monoxide (CO) toxicity at a concentration of 12,800 ppm, as extrapolated from the findings of Golden (2008) and the method used by Agoro et al. (2017).

(ii) **Strangulation Group:** Death was induced by manual strangulation, mimicking human homicides where the neck is compressed by hand (Omelianchuk et al., 2022).

The two control groups involved:

1. **Strangulation Control Group:** Rabbits in this group were also subjected to manual strangulation, without any further exposure to CO.
2. **Chloroform Intoxication Group:** The rabbits in this group were exposed to chloroform to induce death, following methods described by Agoro et al. (2017).

The observed agonal periods before death were 20 minutes for the CO-treated group, 10 minutes for the strangulation group, and 27 minutes for the chloroform group. After death, all subjects were left for 24 hours before vitreous humor samples were collected for analysis. The selection of vitreous humor as the biological specimen was based on its stability postmortem, its similarity to blood in biochemical composition, and its minimal

variation due to age or sex (Agoro et al., 2018, 2019, 2020).

2.4 Ethical Considerations

This study adhered to the ethical standards set by the National Institutes of Health (NIH) and the International Council for Laboratory Animal Science (ICLAS). The protocol was reviewed and approved by the Ethics Committee of the Federal University Otuoke, Bayelsa State, before the commencement of the study. Humane methods of animal handling and care were followed, ensuring that animals were housed in comfortable conditions and provided with appropriate food and water throughout the study. Efforts were made to minimize any discomfort, pain, or distress during the experiment. The causes of death, including CO toxicity and strangulation, were chosen based on their relevance to forensic toxicology while adhering to ethical standards for animal research.

2.5 Selection Criteria

Rabbits used in the study were selected based on health status. Only healthy, active rabbits, as confirmed by a veterinary doctor, were included. Rabbits showing signs of illness or abnormal behavior were excluded from the study. Furthermore, any vitreous humor samples that appeared turbid or contaminated with tissue fragments were rejected to maintain the quality of the results.

2.6 Sample Collection

Vitreous humor samples were collected 24 hours after death following the methods outlined by Coe (1993) and Tente (2004). A 5 mL syringe and needle were used to perform a scleral puncture at the lateral canthus of each rabbit's eye, from which approximately 1.0 mL of clear vitreous humor was aspirated. The samples collected were free of tissue contaminants and fragments, ensuring their suitability for biochemical analysis.

2.7 Preparation of Samples

Collected vitreous humor samples destined for glucose concentration measurements were transferred to fluoride oxalate tubes. Those designated for the determination of proteins, electrolytes, uric acid, lipid profiles, and biomarkers were transferred to plain containers. Samples were then centrifuged at 2050 rpm for 10 minutes, and the supernatants were separated and used for biochemical analyses. These analyses included the determination of sodium, potassium, chloride, calcium, bicarbonate, total protein, albumin, globulin, urea, creatinine, glucose, cholesterol, triacylglycerol, uric acid, lactate dehydrogenase (LDH), and creatine kinase (CK) concentrations (Agoro et al., 2021).

2.8 Sample Analysis

The vitreous humor was analyzed for various biochemical parameters using standardized methods. The concentrations of electrolytes (sodium, potassium, chloride, calcium, bicarbonate) were determined using an ion-selective electrode (ISE) analyzer (ISE 4000) as described by Bolarin and Azinge (2010). Total protein was quantified using the Biuret method (Randox Laboratories, UK), while albumin was measured using the Bromocresol Green Method (Randox Laboratories, UK). Globulin concentration was calculated as the difference between total protein and albumin levels. Urea was estimated using the diacetyl monoxime method, creatinine using Jaffe's method, and glucose using the glucose oxidase method (Randox Laboratories, UK). Lipid profiles (cholesterol, triacylglycerol), uric acid, LDH, and CK activities were also measured using kits from Agappe Diagnostics (Switzerland).

2.9 Statistical Analyses

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 18-21 and Microsoft Excel. All biochemical parameters were measured in triplicates, and one-way ANOVA (Post Hoc-LSD) was used to compare the mean values among the experimental groups. The Student's t-test was employed for group comparisons, with statistical significance set at $P < 0.05$. Pearson correlation was used to determine

relationships among the various biochemical parameters.

3.0 Results and Discussion

The postmortem vitreous levels of selected biochemical parameters in rabbits subjected to carbon monoxide (CO) toxicity and those in cases disguised as CO toxicity are summarized in Table 1 (Appendix I) and visualized in Figures 1–11. Statistical analysis revealed that Na, Cl, CO_2, Ca, albumin, glucose, and total cholesterol levels were not significantly different across all groups ($P > 0.05$). However, significant variations were observed in other parameters, as discussed below.

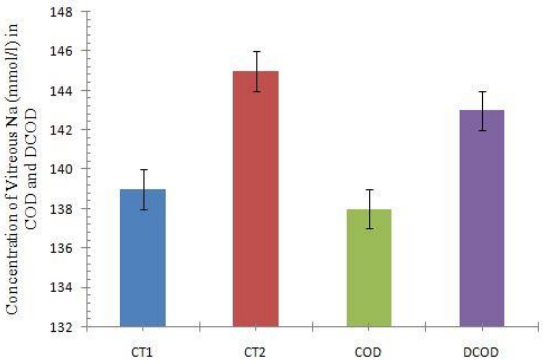

Fig. 1: Chart showing postmortem vitreous levels of Na in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)

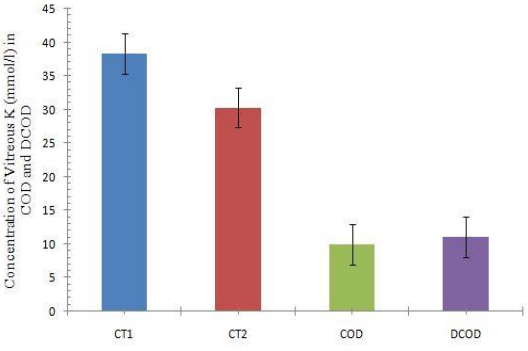

Fig. 2: Chart showing postmortem vitreous levels of K in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)

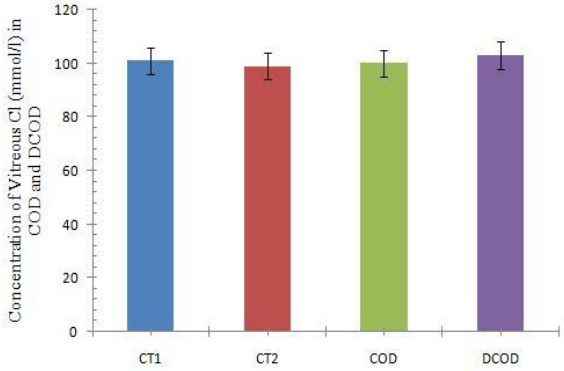

Fig. 3: Chart showing postmortem vitreous levels of Cl in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)

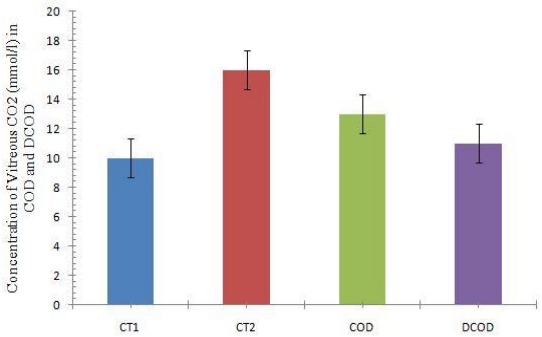

Fig. 4: Chart showing postmortem vitreous levels of CO_2 in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)

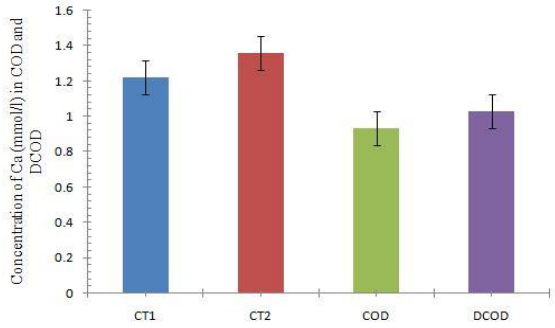

Fig. 5: Chart showing postmortem vitreous levels of Ca in COD and DCOD
CT1: Control 1 (Strangulation death),

CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)

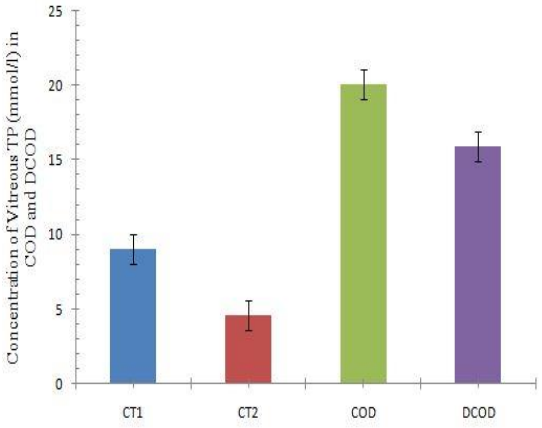

Fig. 6: Chart showing postmortem vitreous levels of total protein in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
TP: Total protein

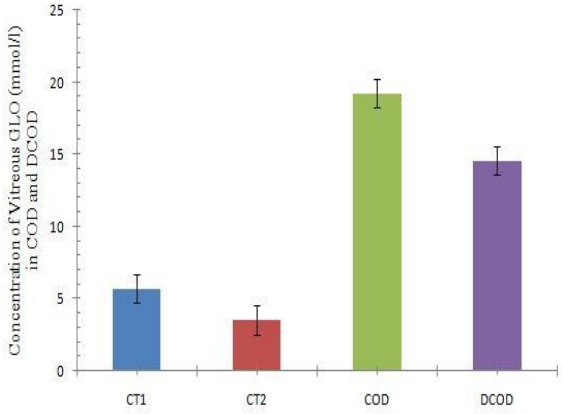

Fig. 7: Chart showing postmortem vitreous levels of globulin in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
GLO: Globulin

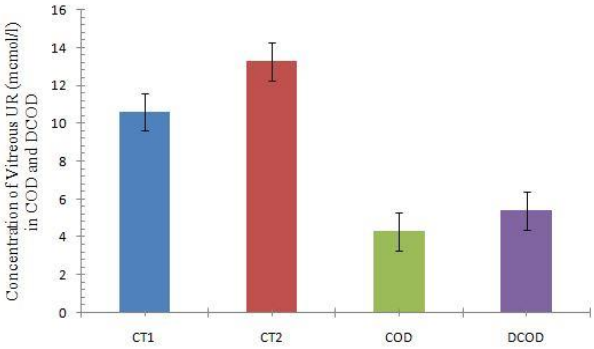

Fig. 8: Chart showing postmortem vitreous levels of urea in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
UR: Urea

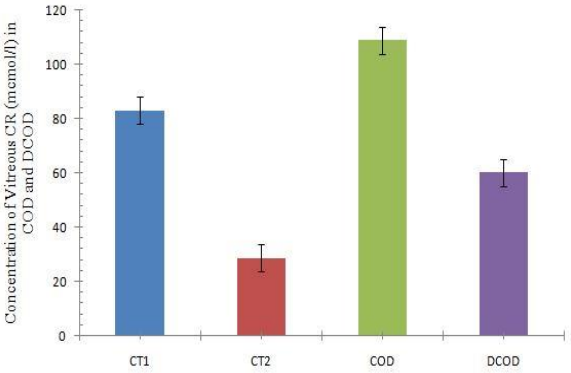

Fig. 9: Chart showing postmortem vitreous levels of creatinine in COD and DCOD
CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
CR: Creatinine

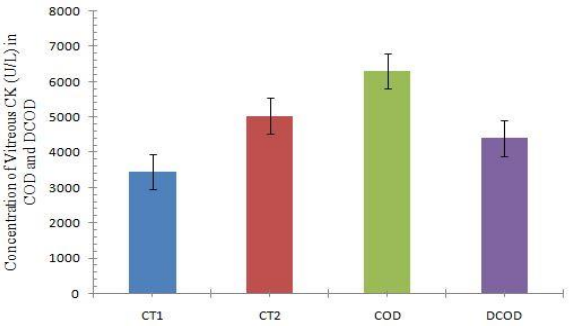

Fig. 10: Chart showing postmortem vitreous levels of creatine kinase in COD and DCOD

CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
CK: Creatine kinase

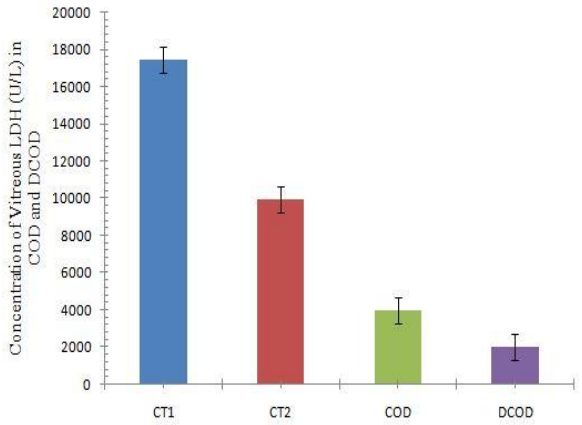

Fig. 11: Chart showing postmortem vitreous levels of lactate dehydrogenase in COD and DCOD

CT1: Control 1 (Strangulation death)
CT2: Control 2 (Chloroform death)
COD: CO intoxication death
DCOD: Disguised CO death (strangled to death before CO exposure)
LDH: Lactate dehydrogenase

The results of this study showed significant variations in the biochemical parameters of postmortem vitreous samples from rabbits exposed to carbon monoxide (CO) toxicity compared to those in control groups. Potassium (K) levels were markedly reduced in the test groups, with values of 9.900 ± 0.04082 mmol/L in the COD group and 11.000 ± 0.4082 mmol/L in the DCOD group, significantly lower than the control groups (38.300 ± 0.04082 mmol/L and 30.300 ± 0.04082 mmol/L, respectively). This decline is consistent with CO-induced metabolic alkalosis, which promotes potassium ion loss as the body compensates by excreting hydrogen ions. This phenomenon aligns with findings from Vasudevan et al. (2019), who linked electrolyte imbalance to CO toxicity.

The study also revealed elevated total protein and globulin levels in the COD and DCOD groups. Total protein levels were 20.110 ± 0.00041 mmol/L in the COD group and 15.880 ± 0.00467 mmol/L in the DCOD group, compared to control values of 9.021 ± 0.00041 mmol/L and 4.613 ± 0.0041 mmol/L, respectively. Similarly, globulin levels increased to 19.220 ± 0.00041 mmol/L in the COD group and 14.550 ± 0.00041 mmol/L in the DCOD group, contrasting sharply with the controls at 5.693 ± 0.00041 mmol/L and 3.500 ± 0.00408 mmol/L. The elevated globulin levels suggest an immune response triggered by CO exposure, as previously observed by Agoro et al. (2017, 2020), highlighting the systemic inflammation and stress response induced by CO toxicity. Triacylglycerol levels were notably elevated in the COD group, reaching 1.060 ± 0.00041 mmol/L, significantly higher than the DCOD group (0.200 ± 0.00041 mmol/L) and control groups (0.010 ± 0.000 mmol/L and 0.311 ± 0.000 mmol/L). This hyperlipidemic response aligns with the metabolic disruptions caused by CO exposure, as described by Petrick et al. (2016) and Simonson et al. (2021), emphasizing the impact of CO on lipid metabolism.

Interestingly, urea levels were significantly reduced in the test groups. The COD group recorded urea levels of 4.300 ± 0.00041 µmol/L, while the DCOD group had levels of 5.401 ± 0.00041 µmol/L, both significantly lower than the controls at 10.617 ± 0.00041 µmol/L and 13.275 ± 0.00041 µmol/L. This reduction might indicate enhanced renal function or altered nitrogen metabolism following CO exposure, consistent with the findings of Csongradi et al. (2012) and Wang et al. (2015). Creatinine levels showed a contrasting trend, with the COD group displaying the highest levels at 109.000 ± 0.40825 µmol/L, followed by the DCOD group at 60.320 ± 0.00041 µmol/L. The controls recorded values of 83.002 ± 0.00041 µmol/L and 28.663 ± 0.00041 µmol/L, respectively. This elevation suggests impaired renal clearance of creatinine, likely due to CO-induced hypotension and reduced perfusion of the kidneys, as supported by studies from Gozubuyuk et al. (2017) and Lee

et al. (2017). Further analysis revealed significantly elevated uric acid levels in the COD group (24.100 ± 0.02283 mmol/L), compared to the DCOD group (49.330 ± 0.00041 mmol/L) and controls (0.632 ± 0.00041 mmol/L and 0.015 ± 0.00041 mmol/L). The elevated uric acid levels highlight the oxidative stress and metabolic disruptions associated with CO toxicity.

Additionally, creatine kinase activity was significantly higher in the COD group at 6310.000 ± 0.40825 U/L, compared to the DCOD group at 4401.000 ± 0.40825 U/L. Control groups showed lower activity levels of 3456.511 ± 0.00041 U/L and 5035.685 ± 0.00041 U/L. The increased activity of this enzyme reflects muscle damage, as CO exposure induces hypoxia and cellular damage. Conversely, lactate dehydrogenase (LDH) activity was reduced in the DCOD group, recorded at 2011.000 ± 0.40825 U/L, compared to the COD group at 4012.000 ± 0.40825 U/L. Control groups showed even higher values of 17491.880 ± 0.00408 U/L and 9948.605 ± 0.00041 U/L. This decline in LDH activity might indicate differing metabolic impacts between direct CO exposure and simulated toxicity.These findings underscore the profound metabolic and biochemical disruptions caused by CO toxicity, with significant implications for understanding its pathophysiological effects and improving diagnostic accuracy in forensic investigations.

4.0 Conclusion

The study aimed to investigate the potential of selected vitreous biochemical parameters as biomarkers for resolving disputed causes of death, specifically in cases of drowning and deaths disguised as drowning, using animal models. The analysis of vitreous humor samples from rabbits subjected to various causes of death revealed significant differences in the biochemical markers across the experimental groups. Notable differences were observed in the levels of sodium, potassium, chloride, calcium, total protein, creatinine, creatine kinase, and lactate dehydrogenase. These findings suggest that vitreous biochemical parameters have the potential to serve as reliable markers for distinguishing between death caused by actual drowning and deaths disguised as drowning, providing valuable support in forensic investigations.

In conclusion, the study demonstrates that the biochemical analysis of vitreous humor could play a critical role in resolving disputes in forensic cases, particularly in distinguishing between different causes of death. The results underscore the importance of further exploring vitreous humor as an alternative to blood samples, which are often unsuitable for postmortem analysis due to decomposition. This method could offer a more accurate and feasible approach in postmortem diagnostics, enhancing the reliability of forensic investigations. It is recommended that future research should focus on expanding the range of biochemical markers analyzed in vitreous humor and validating these findings in human forensic cases. Additionally, the development of standardized protocols for vitreous humor analysis could improve the consistency and applicability of this technique in forensic settings, ultimately contributing to more precise and effective postmortem evaluations in legal contexts. Furthermore, the integration of these findings into forensic practice could help in reducing the exploitation of drowning as a cover for homicide, thereby strengthening the justice system and improving crime resolution.

5.0 References

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Ethical Consideration

The ethical considerations for this study were carefully observed to ensure compliance with national and international guidelines for the

humane treatment of animals used in research. The research protocol was reviewed and approved by the Ethics Committee of the Federal University Otuoke, Bayelsa State, ensuring that the study met ethical standards in line with the *National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals* and the *International Council for Laboratory Animal Science (ICLAS) Ethical Code*.

The study employed humane methods in handling and caring for the rabbits. Special attention was given to avoid causing unnecessary stress, pain, or discomfort to the animals during acclimatization, experimental procedures, and sample collection. Rabbits were selected as the model organism due to their physiological and anatomical similarity to humans in the context of the study objectives. The sample size was calculated using Mead's Resource Equation to ensure that the minimum number of animals necessary for reliable results was used, thereby avoiding excessive use of animals. The animals were acclimatized for seven days in appropriate housing conditions before the commencement of the study. They were provided with adequate ventilation, commercial feed, and water *ad libitum* to ensure their physical well-being throughout the experiment. For the experimental design involving the induction of death, standard and ethically approved methods were employed. These methods followed guidelines that minimize pain and distress to the animals. Procedures like CO toxicity and strangulation were conducted under careful supervision to ensure that the processes adhered to ethical standards and were scientifically justified for the research objectives.

The vitreous humor samples were collected 24 hours postmortem in a manner that preserved the integrity of the samples without introducing unnecessary harm to the carcasses. Proper disposal of the animal remains was conducted in accordance with institutional and environmental regulations. The study adhered to the principle of transparency, ensuring that all procedures were documented and made available for review by the ethics committee. Any deviations from the original protocol were reported promptly.

To minimize biases and conflicts of interest during the experimental processes and analysis, the research team ensured that the primary focus remained on generating reliable and reproducible data while upholding animal welfare. By adhering to these ethical considerations, the research ensured a balance between scientific advancement and the humane treatment of animals, aligning with globally accepted ethical standards.

Authors contributions

Dr. C.G. Ikimi conceptualized the work, did the laboratory analysis and wrote the manuscript. Dr. F.U. Umeoguaju conducted the sample collection. Dr. C.J. Ononamadu did the statistical analysis. All authors read and approved the manuscript.

Ethical Approval

Ethical clearance was obtained from the animal research ethics committee of the Nnamdi Azikiwe University, Awka. The Animal Welfare Act of 1985 of the United States of America for research and Institutional Animal Care and Use Committee (IACUC) protocols were stringently adhered to (Benjamin and Jean, 2016).

Conflict of interest

The authors declared no conflict of interests.

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Availability of data and materials

Data would be made available on request.

Appendix I

Table 1: Parameters levels in post mortem vitreous of rabbits in cases of death by CO toxicity and disguised as CO toxicity

S/N	GROUP	PARAMETERS															
		Na (mmol/l)	K (mmol/l)	Cl (mmol/l)	CO_2 (mmol/l)	Ca (mmol/l)	TP (mmol/l)	ALB (mmol/l)	GLO (mmol/l)	GLU (mmol/l)	TC (mmol/l)	UR(mcmol/l)	CR(mcmol/l)	UA (mmol/l)	CK (U/L)	LDH (U/L)	TG (mmol/l)
1	Cont. 1	139.000±0.40825	38.300±0.04082	101.000±0.47871	10.000±0.47871	1.221±0.00041	9.021±0.00041	3.328±0.00041	5.693±0.00041	0.526±0.00041	0.247±0.00041	10.617±0.0041	83.002±0.0041	0.632±0.00041	3456.511±0.0041	17491.880±0.00408	0.010±0.00000
2	Cont. 2	145.000±0.40825	30.300±0.04082	99.000±0.40825	16.000±0.40825	1.359±0.00041	4.613±0.00041	1.113±0.00041	3.500±0.00408	0.264±0.00041	0.101±0.00041	13.275±0.0041	28.663±0.0041	0.015±0.00041	5035.685±0.0041	9948.605±0.0041	0.311±0.00000
3	COD	138.000±0.40825	9.900±0.04082	100.000±0.40825	13.000±0.40825	1.330±0.00041	20.110±0.0041	0.890±0.00041	19.220±0.0041	1.060±0.00041	0.210±0.00041	4.300±0.00041	109.000±0.40825	24.100±0.02283	6310.000±0.40825	4012.000±0.40825	1.060±0.00041
4	DCOD	143.000±0.40825	11.000±0.40825	103.000±0.40825	11.000±0.40825	1.310±0.00271	15.880±0.0467	1.330±0.00041	14.550±0.0041	0.330±0.00041	0.550 0.00041±	5.410±0.00041	60.320±0.0041	49.330±0.0041	4401.000±0.40825	2011.000±0.40825	0.200±0.00041

Cont. 1: Strangulation death Cont. 2: Chloroform death COD: CO intoxication death DCOD: Disguised CO death (strangled to death before CO exposure) TP: Total protein ALB: Albumin GLO: Globulin GLU: Glucose CR: Creatinine TC: Total cholesterol TG: Triacylglycerol CK: Creatine kinase LDH: Lactate dehydrogenase UR: Urea UA: Uric acid