In ovo and *in vitro* exposure of Paracetamol: Investigation on Vitelline circulation of Chick embryo

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "***In ovo* and *in vitro* exposure of Paracetamol: Investigation on Vitelline circulation of Chick embryo**"is based on the results of investigations carried out by me in the Zoology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Shanti N. Dessai and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report **"*In ovo* and *in vitro* exposure of Paracetamol: Investigation on Vitelline circulation of Chick embryo"** is a bonafide work carried out by **Ms. Divya Laxman Gaude** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Zoology at the School of Biological Sciences and Biotechnology Goa University.

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PREFACE

Embryonic development is a complex and meticulously orchestrated process crucial for the formation of a healthy organism. During this period, a multitude of factors, both intrinsic and extrinsic, influence the growth and differentiation of embryonic tissues, laying the foundation for the individual's future physiology and anatomy. Among these factors, pharmaceutical agents administered during pregnancy have garnered significant attention due to their potential to impact embryonic development.

Paracetamol, also known as acetaminophen, is one such widely used pharmaceutical agent known for its analgesic and antipyretic properties. Its availability over the counter and perceived safety profile have made it a popular choice for managing pain and fever during pregnancy. However, emerging evidence suggests a possible link between prenatal paracetamol exposure and adverse developmental outcomes in offspring, raising concerns regarding its safety during gestation.

This study delves into the intricate relationship between paracetamol exposure and embryonic development, aiming to elucidate the potential effects of this commonly used medication on key aspects of embryogenesis. Through a comprehensive examination of chick embryos exposed to varying doses of paracetamol, we sought to uncover potential disruptions in embryonic growth, cardiovascular function, and vascular morphology.

The preface outlines the rationale for investigating the safety of paracetamol during embryonic development. This research aims to uncover how paracetamol may affect embryonic growth and inform clinical and public health decisions regarding its use in pregnancy. It invites readers to join in exploring the intersection of scientific inquiry and real-world implications, hoping to deepen understanding of prenatal drug exposure's effects on future generations. Ultimately, the study aims to promote maternal and fetal health amidst evolving pharmacotherapy and reproductive medicine practices.

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Abbrevations used

Abbrevations	Full forms
AVV	Anterior vitelline vein
PVV	Posterior vitelline vein
APVV	Anterior and Posterior vitelline veins
RVA	Right vitelline artery
RLVV	Right lateral vitelline vein
LVA	Left vitelline artery
LLVV	Left lateral vitelline vein

ABSTRACT

Investigation of the effects of paracetamol exposure on embryonic development, focusing on embryo length, heart rate, and vitelline vessel morphology, is presented here. The study aims to understand the potential impacts of paracetamol, a commonly used analgesic, on key aspects of embryonic growth and cardiovascular function. Through a series of experiments involving chick embryos exposed to varying doses of paracetamol, embryo length, heart rate, and vitelline vessel diameter were measured at different developmental time points. The methodology involved monitoring these parameters across control and treatment groups and analysing the data for trends and significant differences. The results reveal a dose-dependent effect of paracetamol on embryo length, with higher doses associated with reduced growth rates. Fluctuations in heart rate across dosage groups and time points suggest a potential influence of paracetamol on embryonic cardiac activity. Furthermore, significant alterations in vitelline vessel diameter indicate disruptions in vitelline circulation following paracetamol exposure. These findings underscore the complexity of paracetamol's effects on embryonic development and cardiovascular function, highlighting the need for further research to elucidate underlying mechanisms and potential implications for fetal health. Overall, this study contributes valuable insights into the safety profile of paracetamol during pregnancy and informs ongoing discussions regarding its use in clinical practice.

Keywords: Paracetamol, Embryonic development, Chick embryos, Heart rate, Vitelline vessels, Dose-dependent effect.

INTRODUCTION

CHAPER 1: INTRODUCTION

1.Introduction

Paracetamol represents a versatile and widely utilized pharmaceutical compound with well-established analgesic and antipyretic properties. Its chemical composition, pharmacological profile, modes of administration, and safety considerations render it an ideal candidate for investigating its effects on embryonic development and circulatory function in experimental models such as chick embryos. By elucidating the mechanisms underlying Paracetamol's pharmacological actions and potential developmental impacts, ongoing research endeavors aim to inform clinical practice, advance drug discovery efforts, and enhance our understanding of embryonic physiology and pathophysiology.

1.1.Background

The circulatory system of the chick embryo comprises several vitelline vessels that play crucial roles in transporting nutrients, oxygen, and waste products during embryonic development:

Anterior and Posterior Vitelline Vein (APVV): The anterior and posterior vitelline veins are major vessels responsible for carrying nutrient-rich blood away from the yolk sac towards the developing embryo. The anterior vitelline vein typically emerges from the area vasculosa and travels anteriorly, while the posterior vitelline vein originates from the sinus terminalis and courses posteriorly. Together, these veins form an essential part of the vitelline circulation, facilitating the exchange of essential substances between the yolk sac and the developing embryo.

Left Lateral Vitelline Vein (LLVV): The left lateral vitelline vein is another significant vessel in the chick embryo's circulatory system, responsible for transporting blood away from the yolk sac. It branches off from the anterior or posterior vitelline vein and courses laterally towards

the embryo. The LLVV contributes to the distribution of nutrients and oxygen to different regions of the developing chick embryo.

Left and Right Vitelline Arteries (LVA, RVA): The left and right vitelline arteries are major vessels that carry deoxygenated blood from the embryo back to the yolk sac for nutrient replenishment. These arteries typically originate from the developing embryo and course towards the yolk sac, forming an essential part of the vitelline circulation loop. The LVA and RVA function in conjunction with the vitelline veins to maintain the flow of blood and ensure the efficient exchange of substances necessary for embryonic growth and development. These vitelline vessels collectively form a sophisticated circulatory network in the chick embryo, facilitating the exchange of essential nutrients, oxygen, and metabolic waste products between the developing embryo and the yolk sac.

Chick embryos have long served as a valuable model system for studying vertebrate embryonic development, offering insights into fundamental biological processes that are conserved across species, including humans. Additionally, paracetamol, a widely used medication during pregnancy, presents an interesting issue to explore because of its potential impact on embryonic development and the associated implications for maternal and fetal health.

Understanding how paracetamol exposure influences the vitelline circulation of chick embryos is of particular interest due to the critical role of the circulatory system in supporting embryonic growth and organogenesis. The vitelline circulation serves as the primary means of nutrient and oxygen delivery to the developing embryo during early stages of development, making it essential for normal embryonic development. Disruption of this circulation by paracetamol could have profound implications for embryonic growth and viability.

Moreover, investigating the effects of paracetamol exposure on chick embryos provides an opportunity to elucidate the mechanisms underlying its teratogenic potential. While paracetamol is generally considered safe when used at recommended doses, concerns have been

raised regarding its safety during pregnancy, particularly its potential to induce developmental abnormalities. By studying its effects on chick embryos, researchers can gain insights into the mechanisms through which paracetamol may exert its teratogenic effects and identify potential targets for intervention.

Furthermore, this research has important implications for clinical practice, as paracetamol is commonly used by pregnant women for pain relief and fever management. Understanding the safety profile of paracetamol during pregnancy is essential for informing clinical guidelines and recommendations for its use in pregnant women. By exploring the effects of paracetamol exposure on chick embryos, researchers can contribute valuable data to the ongoing debate surrounding the safety of paracetamol use during pregnancy and help guide clinical decision-making.

In the past, the relevance of investigating the potential changes in vitelline circulation induced by paracetamol was underscored by its widespread use and potential impact on embryonic development. Paracetamol, a commonly used pharmaceutical agent known for its analgesic and antipyretic properties, raised concerns regarding its safety during pregnancy due to its potential to cross the placental barrier and reach the developing embryo. Given the critical role of vitelline circulation in supplying nutrients and oxygen to the developing chick embryo, alterations in this circulatory system were hypothesized to reflect underlying disruptions in embryonic vascular development and functionality induced by paracetamol exposure. Thus, elucidating the effects of paracetamol on vitelline circulation was deemed essential for understanding its potential teratogenic effects and informing clinical practices surrounding its use in pregnant women.

1.2. Problem Statement:

The widespread use of paracetamol (acetaminophen) during pregnancy raises concerns about its potential adverse effects on embryonic development, particularly regarding the vitelline circulation of the chick embryo. Despite its common use as an analgesic and antipyretic medication, emerging evidence suggests that paracetamol exposure during pregnancy may disrupt normal vascular development in the embryo, leading to cardiovascular abnormalities and other developmental defects Bauer *et al*. (2021) & Bremer *et al*. (2017). This issue is compounded by the lack of clear regulatory guidelines and public awareness regarding the safe use of paracetamol during pregnancy, posing a significant risk to maternal and fetal health.

1.3. Possible Solutions:

Regulatory Guidelines: Implementing stringent regulatory guidelines for the use of paracetamol during pregnancy, including standardized recommendations on dosage, frequency, and duration of use, to mitigate potential teratogenic effects on embryonic vascular development.

Public Awareness Campaigns: Launching targeted public awareness campaigns to educate pregnant women and healthcare providers about the potential risks associated with paracetamol use during pregnancy, emphasizing the importance of informed decision-making and consulting healthcare professionals before initiating any medication regimen.

Alternative Pain Management: Promoting the exploration and adoption of alternative pain management strategies for pregnant women, such as non-pharmacological interventions (e.g., physical therapy, acupuncture, mindfulness techniques) and safer analgesic options with minimal teratogenic potential, to reduce reliance on paracetamol.

Enhanced Clinical Monitoring: Advocating for enhanced clinical monitoring of pregnant women who require paracetamol treatment, including regular prenatal check-ups, fetal

ultrasound examinations, and cardiovascular assessments, to detect any potential developmental abnormalities early and facilitate timely intervention.

Research and Development: Investing in research and development efforts to identify safer analgesic alternatives for pregnant women, including the development of novel pharmacological agents and non-invasive pain relief modalities tailored specifically for use during pregnancy, to address the unmet need for effective pain management while minimizing fetal risks.

1.4.The aim of the study

The aim of this study is to investigate the effects of *in ovo* and *in vitro* exposure to paracetamol on the vitelline circulation of the chick embryo, assessing alterations in vascular development and functionality.

1.5.The objectives of the study

- To assess the impact of a single *in ovo* Paracetamol exposure in the air sac on vitelline circulation in chick embryos, with observations at specified time intervals (48, 72, 96, and 120 hours of incubation).
- To investigate the influence of Paracetamol application on vitelline circulation in opened chick embryos through *in vitro* studies.
- To examine heart rate of Chick Embryos under the influence of paracetamol.

1.6.Research questions

Based on the objectives here are the corresponding research questions:

- How does *in ovo* exposure to Paracetamol affect the vitelline circulation in chick embryos over different developmental time points?
- What are the effects of *in vitro* Paracetamol application on vitelline circulation in opened chick embryos at different developmental time period?
- How does Paracetamol exposure influence heart rate in chick embryos during early developmental stages?
- How do different doses of *in ovo* Paracetamol exposure affect the morphology and functionality of vitelline vessels in chick embryos over distinct developmental stages?
- What are the underlying mechanisms through which Paracetamol alters vitelline circulation in opened chick embryos, as observed *in vitro*, and how do these effects compare to *in ovo* exposure?
- Are there dose-dependent effects of Paracetamol on heart rate in chick embryos, and does this influence vary across different developmental stages?

1.7.Hypotheses of the study

Hypotheses of this study propose that *in ovo* Paracetamol exposure alters the development of vitelline vessels in chick embryos. *In vitro* Paracetamol application is expected to mirror these effects, indicating a direct influence on circulation dynamics. Specifically, Paracetamol exposure is hypothesized to minimally affect heart rate, underscoring its primary vascular impact.

1.8.Conspectus of the study

The study unfolded through three distinct phases aimed at unravelling the effects of Paracetamol exposure on the vitelline circulation of chick embryos. Initially, a comprehensive assessment was conducted through *in ovo* exposure, scrutinizing the impact of a single Paracetamol dose on vitelline circulation at pivotal developmental intervals (48, 72, 96, and 120 hours of incubation). Subsequently, *in vitro* studies were pursued to delve deeper into the influence of Paracetamol application on vitelline circulation in opened chick embryos. Additionally, hematological parameters, including heart rate, were meticulously examined to elucidate the broader implications of Paracetamol exposure on circulatory function and embryonic well-being. Through these structured phases, the study aimed to provide critical insights into the intricate relationship between Paracetamol exposure and vitelline circulation dynamics during embryonic development.

LITERATURE

REVIEW

CHAPTER 2: LITERATURE REVIEW

2. Literature review

Several studies have been conducted to explore the effects of Paracetamol exposure on embryonic development and circulatory function, providing valuable insights into the potential risks and mechanisms involved. For instance, research by Haider Ali (2022) delved into the stages of chick embryo development, offering foundational knowledge crucial for understanding the effects of external factors like Paracetamol. Similarly, investigations by ElMazoudy and Bekhet (2016) explored the teratological effects of aluminium on chick embryo heart and vascularization, shedding light on the susceptibility of embryonic circulatory systems to environmental toxins. Additionally, studies by Lis *et al*. (2006) and Blecharz-Klin *et al*. (2015, 2017) investigated the effects of Paracetamol on embryonic development and neurological function in various animal models, providing valuable insights into potential mechanisms and outcomes. Furthermore, the work of Bremer *et al*. (2017) and Kilcoyne and Mitchell (2017) examined the impact of Paracetamol exposure during pregnancy on hematopoietic stem cell populations and male reproductive development, respectively, highlighting potential implications for fetal health. Collectively, these studies contribute to the growing body of literature aimed at elucidating the effects of Paracetamol exposure on embryonic development and circulatory function, providing a foundation for further research in this area.

2.1. Published literature on the studies already carried out to find the solution to the problem as presented in this research undertaken.

Several studies have been conducted to explore the effects of Paracetamol exposure on embryonic development and circulatory function, providing valuable insights into the potential risks and mechanisms involved. For instance, research by Haider ali (2022) delved into the stages of chick embryo development, offering foundational knowledge crucial for understanding the effects of external factors like Paracetamol. Similarly, investigations by ElMazoudy and Bekhet (2016) explored the teratological effects of aluminium on chick embryo heart and vascularization, shedding light on the susceptibility of embryonic circulatory systems to environmental toxins. Additionally, studies by Lis *et al*. (2006) and Blecharz-Klin *et al*. (2015, 2017) investigated the effects of Paracetamol on embryonic development and neurological function in various animal models, providing valuable insights into potential mechanisms and outcomes. Furthermore, the work of Bremer *et al*. (2017) and Kilcoyne and Mitchell (2017) examined the impact of Paracetamol exposure during pregnancy on hematopoietic stem cell populations and male reproductive development, respectively, highlighting potential implications for fetal health. Collectively, these studies contribute to the growing body of literature aimed at elucidating the effects of Paracetamol exposure on embryonic development and circulatory function, providing a foundation for further research in this area.

Additional studies have investigated the developmental effects of various substances on chick embryos, offering valuable insights into potential teratogenic mechanisms. For example, research by Khosravi *et al*. (2018) explored the embryotoxic effects of meglumine antimoniate using a chick embryo model, highlighting the sensitivity of embryonic tissues to chemical insults. Moreover, investigations by Lis *et al*. (2006) assessed the impact of Paracetamol injection on thyroid hormone levels in chicken embryos, providing evidence of endocrine

disruption during critical developmental stages. Furthermore, studies by De Melo Bernardo *et al*. (2012) and Vergara and Canto-Soler (2012) focused on the migration and development of primordial germ cells and retinal tissue in chick embryos, respectively, offering insights into the broader effects of environmental factors on embryonic patterning and differentiation. Additionally, work by Moungmaithong *et al*. (2022) evaluated the morphological effects of aspirin, ibuprofen, and Paracetamol exposure using a whole embryo culture system, highlighting the potential for *in vitro* models to complement *in ovo* studies in assessing developmental toxicity. Overall, these studies contribute to our understanding of the complex interactions between external factors, embryonic development, and circulatory function in avian models, providing essential context for the present research endeavor.

For instance, ElMazoudy and Bekhet (2016) delved into the toxicological effects of aluminium on the embryonic chick heart and vascularization, shedding light on potential hazards posed by environmental contaminants. Additionally, studies by Lis *et al*. (2006) investigated the effects of Paracetamol injection on hatching and thyroid hormone levels in chicken embryos, revealing potential endocrine-disrupting properties of this widely used pharmaceutical. Furthermore, research by Labba *et al*. (2022) examined the neurotoxic effects of Paracetamol exposure on neuronal arborization and cytoskeletal proteins in both human and chicken *in vitro* models, providing insights into the molecular mechanisms underlying its developmental toxicity. Moreover, investigations by Haider Ali studied the stages of chick embryo development, providing a foundational understanding of avian embryogenesis crucial for interpreting experimental findings. These studies collectively contribute to a comprehensive understanding of the factors influencing chick embryo development, informing the design and interpretation of the current research on Paracetamol's effects on vitelline circulation.

2.2. A critical review of these studies and authors view either supporting or opposing or describing limitations

The existing literature provides valuable insights into the effects of various substances on chick embryo development, particularly concerning Paracetamol exposure. Studies by ElMazoudy and Bekhet (2016), Lis *et al*. (2006), and Labba *et al*. (2022) have each contributed uniquely to our understanding, albeit with varying perspectives and limitations.

ElMazoudy and Bekhet's (2016) investigation into aluminium's toxicological effects on chick embryonic heart and vascularization highlights the potential risks associated with environmental contaminants. While their findings shed light on the hazards posed by aluminium exposure, the extrapolation of these results to Paracetamol's effects may be limited due to differences in chemical properties and mechanisms of action between the two substances.

Similarly, Lis *et al*. (2006) explored the impact of Paracetamol injection on hatching and thyroid hormone levels in chicken embryos, revealing potential endocrine-disrupting properties. However, the study's focus on thyroid hormone levels may not fully capture Paracetamol's broader effects on embryonic development, warranting further investigation into additional developmental parameters.

Labba *et al*. (2022) investigated the neurotoxic effects of Paracetamol exposure on neuronal arborization and cytoskeletal proteins, providing insights into its molecular mechanisms of developmental toxicity. While their findings contribute to understanding Paracetamol's neurotoxic effects, extrapolating these results to broader developmental outcomes requires caution due to the complexity of embryonic development and the multitude of factors involved.

While these studies offer valuable contributions to our understanding of Paracetamol's effects on chick embryo development, each has its limitations. Further research is needed to

comprehensively assess Paracetamol's impact on vitelline circulation and other aspects of embryonic development, considering the diverse array of factors influencing developmental outcomes.

2.3.Previous studies grouped by association of ideas in various paragraphs

The existing literature on chick embryo development and the effects of chemical exposure is vast and diverse, encompassing studies that investigate a wide range of substances and their potential impact on embryonic growth and viability. Within this body of research, studies are often grouped by their association of ideas, focusing on similar mechanisms, endpoints, or experimental approaches.

Toxicological Effects of Pharmaceutical Compounds: Studies exploring the toxicological effects of pharmaceutical compounds on chick embryo development reveal insights into potential hazards posed by environmental contaminants. ElMazoudy and Bekhet (2016) investigated the impact of aluminium exposure on chick embryonic heart and vascularization, while Lis *et al*. (2006) explored Paracetamol's effects on hatching and thyroid hormone levels in chicken embryos. These studies contribute to understanding how pharmaceutical substances may influence embryonic development and viability.

Neurotoxic Effects on Chick Embryo Neuronal Development: Research into the neurotoxic effects of chemical exposure on chick embryo neuronal development sheds light on molecular mechanisms of developmental toxicity. Labba *et al*. (2022) examined Paracetamol's impact on neuronal arborization and cytoskeletal proteins, highlighting the importance of understanding chemical impacts on neural development during embryogenesis.

Effects on Specific Developmental Parameters: Studies focused on specific developmental parameters or endpoints provide valuable insights into teratogenic effects and developmental

mechanisms. Tufan *et al*. (2007) investigated ethanol's effects on chick embryo optic nerve development. These studies elucidate mechanisms underlying cardiac and craniofacial teratogenesis.

2.4.Scope of the study

The scope of this research study encompasses a comprehensive investigation into the effects of *in ovo* and *in vitro* exposure to Paracetamol on the vitelline circulation of chick embryos. The study will focus on assessing alterations in vascular development and functionality induced by Paracetamol administration at various stages of embryonic development. Key aspects of the research scope include:

Evaluation of Vitelline Circulation: The study will assess the impact of Paracetamol exposure on the vitelline circulation system of chick embryos, including changes in vessel morphology, diameter, and blood flow dynamics. Observations will be conducted at specified time intervals (48, 72, 96, and 120 hours of incubation) to capture developmental changes and acute effects of Paracetamol.

Comparative Analysis: The research will compare the effects of Paracetamol exposure between *in ovo* and *in vitro* experimental models to elucidate differences in response and potential mechanisms of action. By examining vitelline circulation in both experimental settings, the study aims to provide a comprehensive understanding of Paracetamol's impact on embryonic vascular development.

Evaluations of Heart rate changes: This analysis will encompass measurements of heart rate, to evaluate systemic effects of Paracetamol exposure on embryonic circulation.

Developmental Stage Considerations: The research will consider the influence of embryonic developmental stage on the susceptibility to Paracetamol-induced effects. By conducting observations at multiple time points during chick embryo incubation, the study aims to delineate critical windows of vulnerability and developmental milestones that may impact the response to Paracetamol exposure.

Implications for Human Health: While the study focuses on chick embryos as a model system, findings may have implications for human health, particularly regarding the safety of Paracetamol use during pregnancy.

METHODOLOGY

CHAPTER 3: METHODOLOGY

3.1. Paracetamol-Test Compound

Paracetamol, also known by its chemical name acetaminophen, stands as one of the most commonly used over-the-counter medications worldwide, celebrated for its analgesic and antipyretic properties. Its widespread application in clinical settings and households alike underscores its pivotal role in managing pain and fever across diverse populations. Despite its ubiquity, the intricate mechanisms underlying its pharmacological actions and potential effects on developing organisms remain subjects of ongoing research and debate. This comprehensive description aims to elucidate the chemical composition, pharmacological properties, modes of administration, and safety considerations associated with Paracetamol, the test compound employed in the study investigating its impact on vitelline circulation in chick embryos.

Chemical Composition: Paracetamol, chemically designated as N-(4-hydroxyphenyl) acetamide, boasts a straightforward molecular structure composed of a hydroxyl-substituted aromatic ring linked to an acetamide functional group. Its chemical formula, $C_8H_9NO_2$, delineates its elemental composition, comprising carbon, hydrogen, nitrogen, and oxygen atoms in precise stoichiometric ratios. This molecular arrangement imparts Paracetamol with its characteristic properties, facilitating interactions with biological targets implicated in pain perception and temperature regulation.

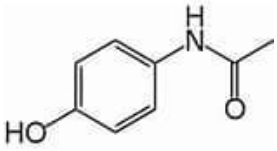

Figure 3.1 Chemical structure of Paracetamol ($C_8H_9NO_2$)

Pharmacological Properties: The pharmacological profile of Paracetamol primarily revolves around its action as an analgesic and antipyretic agent, exerting its effects through multiple mechanisms within the central nervous system (CNS). Unlike nonsteroidal anti-inflammatory drugs (NSAIDs), which primarily inhibit cyclooxygenase (COX) enzymes, Paracetamol's precise mode of action remains incompletely understood. While early hypotheses posited its involvement in COX inhibition, subsequent research has implicated the endocannabinoid system, serotonergic pathways, and nitric oxide signaling in its analgesic effects. Paracetamol's antipyretic action is thought to involve modulation of hypothalamic thermoregulatory centres, leading to reduced fever responses without affecting basal body temperature. Additionally, Paracetamol exhibits minimal anti-inflammatory activity, distinguishing it from traditional NSAIDs and making it a preferred option for individuals sensitive to gastrointestinal or cardiovascular side effects associated with COX inhibition.

Modes of Administration: Paracetamol is available in various formulations tailored to diverse patient populations and clinical needs. Oral tablets, capsules, and liquid suspensions represent the most common routes of administration, offering convenience and ease of dosing for both adults and children. Additionally, intravenous formulations provide rapid relief for patients unable to tolerate oral medications or experiencing acute pain or fever. In the context of experimental research, Paracetamol can be administered via *in ovo* injection into the air sac of developing chick embryos or through *in vitro* exposure using tissue culture techniques. These methodologies enable precise control over dosage and exposure duration, facilitating the investigation of Paracetamol's effects on embryonic development and circulatory function under controlled experimental conditions.

Safety Considerations: While Paracetamol is generally regarded as safe when used as directed, certain precautions and considerations apply to its clinical and experimental use. Overdose or misuse of Paracetamol can lead to hepatotoxicity, highlighting the importance of

adhering to recommended dosage regimens and avoiding concomitant use with other hepatotoxic substances such as alcohol. In experimental settings, careful attention must be paid to dosage selection, route of administration, and monitoring of potential adverse effects on experimental subjects. Furthermore, ethical considerations regarding animal welfare and the 3Rs (Replacement, Reduction, Refinement) framework should guide experimental design and conduct to minimize harm and maximize scientific validity.

Paracetamol represents a versatile and widely utilized pharmaceutical compound with well-established analgesic and antipyretic properties. Its chemical composition, pharmacological profile, modes of administration, and safety considerations render it an ideal candidate for investigating its effects on embryonic development and circulatory function in experimental models such as chick embryos. By elucidating the mechanisms underlying Paracetamol's pharmacological actions and potential developmental impacts, ongoing research endeavors aim to inform clinical practice, advance drug discovery efforts, and enhance our understanding of embryonic physiology and pathophysiology.

3.2. Experimental model chosen for the study

The chick embryo serves as an excellent experimental model for studying the effects of Paracetamol exposure on embryonic development, particularly regarding vitelline circulation. Several factors contribute to its suitability for this study:

Developmental Similarities: Chick embryos share significant developmental similarities with mammalian embryos, making them valuable models for studying embryonic processes, including vascular development. Their rapid and well-characterized development allows for precise staging and observation of developmental changes.

Accessible Vasculature: The chick embryo's vasculature, including the vitelline circulation, is easily accessible for observation and manipulation. This accessibility enables researchers to directly visualize and analyze vascular changes in response to Paracetamol exposure.

Ex Ovo and *In Ovo* Techniques: Chick embryos can be cultured *ex ovo* (outside the egg) or *in ovo* (inside the egg), providing flexibility in experimental approaches. *In ovo* exposure closely mimics *in vivo* conditions and allows for the study of systemic effects, while *ex ovo* techniques offer better access for imaging and manipulation.

Embryonic Transparency: During early stages of development, chick embryos exhibit transparency, allowing for non-invasive imaging of internal structures, including the vasculature. This transparency facilitates high-resolution imaging techniques such as confocal microscopy and optical coherence tomography.

Ethical Considerations: Chick embryos are not subject to the same ethical constraints as mammalian embryos, making them an attractive model for developmental studies. Their use allows researchers to conduct experiments that may not be feasible or permissible in mammalian models.

Cost-Effectiveness: Chick embryos are relatively inexpensive and easy to obtain compared to mammalian models, making large-scale studies more feasible. This cost-effectiveness allows for the replication of experiments and the exploration of various experimental conditions.

Additionally, chick embryos allow for the development of vitelline circulation, which is crucial for nutrient exchange during early embryonic stages. The experimental setup involved obtaining fertilized eggs (*Gallus gallus*, Srinidhi strain) from a local hatchery and incubating them under controlled conditions.

3.3. Experimental Setup

The experiments commenced after obtaining approval from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) (Approval no. GUZ/IAEC/23-24/N8) for the usage of eggs. Egg collection and incubation involved obtaining fertilized eggs of the Srinidhi Strain (*Gallus gallus domesticus*), sourced from the local hatchery (Goa Institute of Rural Development and Administration, Ela Farm, Old Goa, Goa, 403-402, India). These eggs, with an average weight of 50-55g, underwent viability testing through candling prior to incubation.

Subsequently, the eggs were placed in an incubator set at a temperature of 37°C and a relative humidity of 50-60%, maintaining a horizontal position throughout the incubation period. Day zero (D0) marked the initiation of the incubation process, with Hamburger and Hamilton's (1951) developmental staging used to determine the embryonic age throughout the incubation period.

Paracetamol dose preparation: The preparation of the paracetamol dose involved meticulous calculations based on the human acceptable daily dose, which stands at 4 g per 60 kg body weight. Through mathematical extrapolation, the required dosage for the chick embryos was determined to be 0.06 mg per gram of body weight. Utilizing this formula and considering the average weight of the eggs (55g), the calculated dose amounted to 3.3 mg of paracetamol per egg. Febrinil, the paracetamol injection used in the study, was administered via pipetting, with precise measurements of 0.01 microliter for the 2 mg dose and 0.02 microliter for the 3 mg dose, as detailed by Chumpanya *et al*. (2020).

Experimental groups and *in ovo* injection: A batch of eggs, incubated for 24 hours, was meticulously prepared for experimentation. Prior to the commencement of the study, the eggs were carefully divided into two groups: experimental (E) and control (C), with each group

comprising 20 eggs. To ensure a contamination-free environment, the eggs were gently wiped with 70% ethanol and examined for viability through candling. Subsequently, a small hole was delicately created on the blunt side of each egg using a needle, while the eggs were positioned horizontally along their long axis. With precision, the calculated dose of paracetamol (3.3mg) was administered through the air sac using a 20-microliter pipette. Following injection, the holes were meticulously sealed using paraffin wax to prevent any potential contamination. To facilitate observations at various developmental stages, the injected eggs were further divided into four groups, each comprising five eggs, earmarked for evaluation at 48, 72, 96, and 120 hours of incubation, respectively. Throughout the incubation period, regular candling was performed to assess viability, and any instances of mortality were diligently recorded. In the event of mortality, embryos were carefully extracted to determine their developmental stage and ascertain the presence of any deformities.

Observations at different time period: After reaching specific time intervals, the eggs were carefully removed from the incubator, and their viability was meticulously assessed. Under sterile conditions, the eggs were manually hatched in a glass dish. Utilizing a stereomicroscope, vitelline vessels were observed to monitor changes in vitelline circulation. Photographs of the vessels were captured using a camera attached to the microscope, enabling the measurement of vessel diameter according to the method outlined by Siamwala *et al*. (2013). Furthermore, morphological variations in the heart were meticulously documented, while estimations were made regarding the embryo's age, as well as any observed defects or malposition’s.

Assessment of vitelline vessel morphology: The methodology employed in this study involved careful preparation of samples, wherein fertilized eggs were incubated under controlled conditions until reaching the desired developmental stage. Following aseptic techniques, eggs were delicately opened to expose the chick embryos without causing damage to the vitelline vessels. Subsequently, using a stereo microscope equipped with a camera,

images of the exposed chick embryos were captured from various angles to ensure comprehensive coverage of the vitelline vessels of interest. These images were then transferred to a computer for analysis using image processing software such as Zeiss ZEN, allowing for precise measurements of vessel diameter by outlining them in the images. Diameter measurements of the anterior and posterior vitelline veins (APVV), left lateral vitelline vein (LLVV), left vitelline artery (LVA), and right vitelline artery (RVA) were recorded at predetermined points along the vessels. The observed alterations in vessel diameter were interpreted in the context of paracetamol exposure, developmental stage, and dosage. Finally, conclusions were drawn regarding the effects of paracetamol on vitelline circulation in chick embryos, considering the implications for embryonic development and potential mechanisms of action.

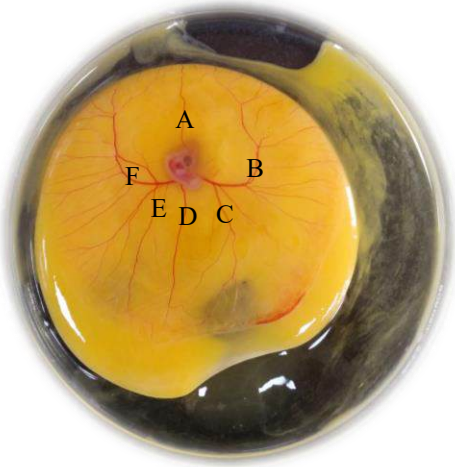

Figure 3.3: Major vitelline vessels of chick embryo (96hrs); **A**: Anterior Vitelline Vein (**AVV**), **B**: Right Vitelline Artery (**RVA**), **C**: Right Lateral Vitelline vein (**RLVV**), **D**: Posterior Vitelline Vein (**PVV**), **E**: Left Vitelline Artery (**LVA**), **F**: Left Lateral Vitelline Vein (**LLVV**)

Heart rate evaluations: To evaluate heart rate, eggs were manually hatched at predetermined intervals and placed in a petri dish. Using a stereomicroscope, the heart of the embryos was observed, and heartbeats were counted for a duration of one minute. This process allowed for the accurate measurement of the heart rate of embryos exposed to paracetamol *in ovo*.

***In vitro* Exposure of Paracetamol**: For *in vitro* paracetamol exposure, the eggshells of eggs at 48, 72 ,96 & 120 hours of incubation were carefully broken under sterile conditions. Prior to exposure, snapshot capturing vascular activity were taken for 0-10 minutes to establish baseline observations. Subsequently, a sterile Whatman No. 1 filter paper disc, soaked in a Paracetamol stock solution of 3mg, was delicately placed on the anterior vein, posterior vein, left and right vitelline artery using sterile forceps. Observations were conducted using a stereomicroscope, with changes in heartbeat upon application of paracetamol meticulously noted. Vessel width was quantified from captured images using Zeiss ZEN 3.9 software, following the methodology outlined by Swaminathan *et al*. (2019).

Heart rate recordings for *In vitro* studies: In terms of heart rate assessment, the eggshells were broken under sterile conditions, and the heartbeat prior to drug exposure was counted. Utilizing sterile forceps, a sterile Whatman No. 1 filter paper disc, saturated with a paracetamol drug solution, was placed on the right vitelline artery. Subsequently, the heartbeat was noted after exposure to each vitelline vessel, ensuring comprehensive observation of any changes in vitelline circulation. Overall snapshots of vitelline circulation were captured to confirm any alterations following exposure.

3.4. Statistical analyses

All other statistical analyses were performed with Microsoft Excel 2010. Data represented for this study are Mean of six samples ± Standard deviation.

- **Collection of fertilized eggs and incubation**

Procurement of fertilized eggs from ICAR (Old Goa)

Candling of eggs

Incubator (37°C , RH- 50%)

Figure 3.3. a) Experimental set up: Primary step of fertilized eggs collection & incubation

- **Injection of Paracetamol *In ovo***

Eggs in incubator (24hrs)

Whipping of eggs with 70% ethanol

Pricking of egg with needle

Injecting dose with pipette

Sealed eggs with paraffin wax

Injected eggs incubation

Figure 3.3.b) Experimental set up: *in ovo* Paracetamol exposure

- **Observation of injected eggs**

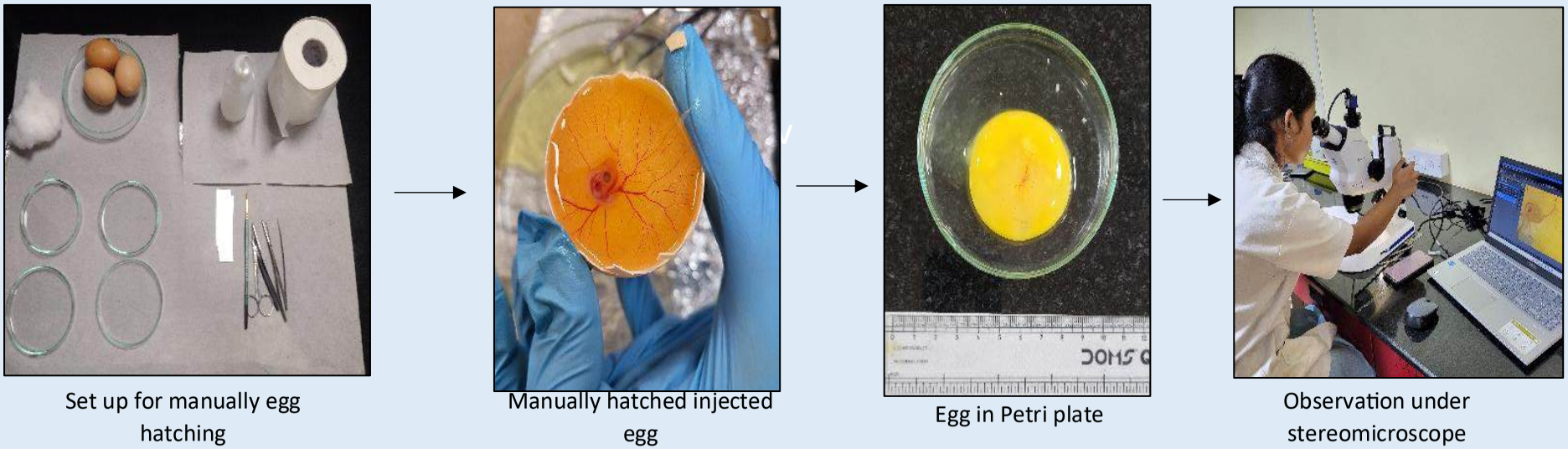

Figure 3.3.c) Experimental setup: Observation of injected eggs under stereomicroscope

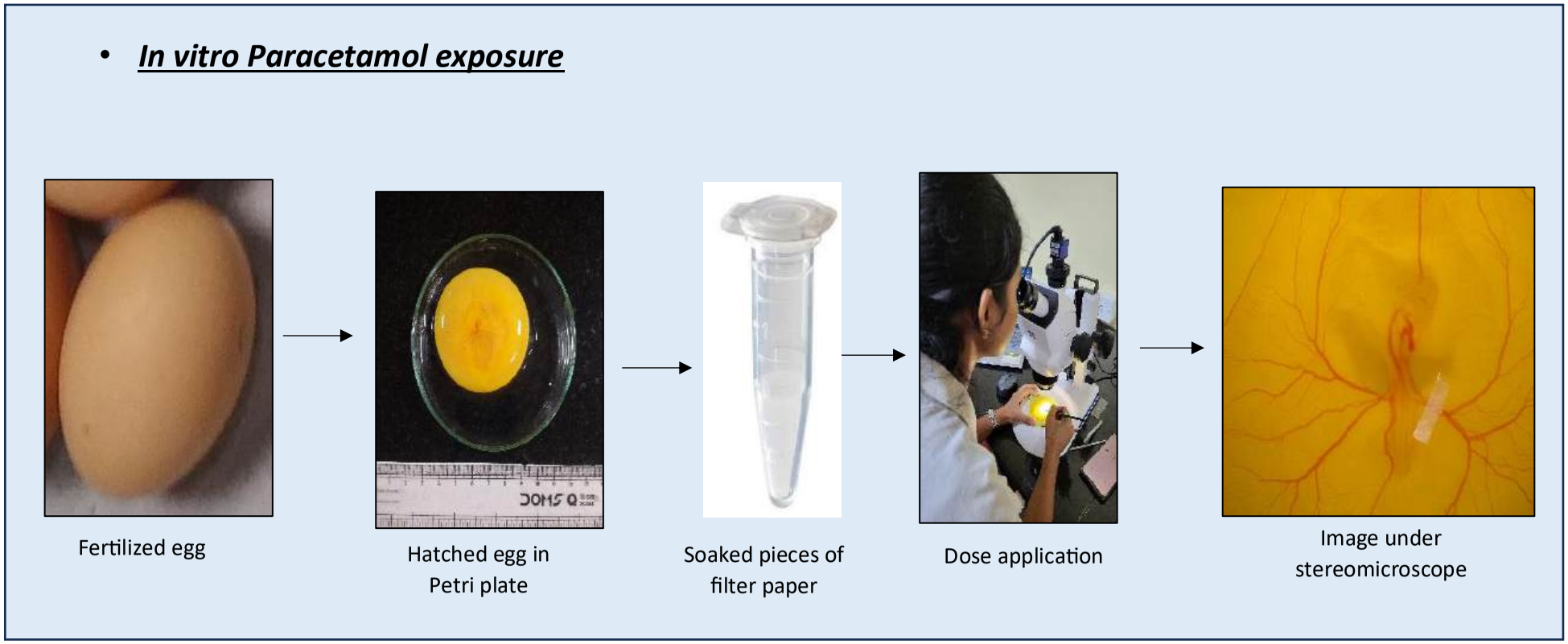

Figure 3.3.d) Experimental set up: *in vitro* Paracetamol exposure procedure

ANALYSES AND

CONCLUSION

CHAPTER 4: ANALYSES & CONCLUSIONS

4.1 Results

4.1.1 Embryo length

Table 4.1.1 presents the mean embryo length at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure.

48 Hours: The control group has the highest mean embryo length (454.51), followed by the 2mg group (426.55), and then the 3mg group (401.53). There's a clear trend of decreasing mean embryo length with increasing paracetamol dosage.

72 Hours: The control group still maintains the highest mean embryo length (529.22), significantly higher than both the 2mg (402.58) and 3mg (523.71) groups. The 2mg group exhibits the lowest mean length at this stage, indicating a potential dose-dependent effect of paracetamol on embryo growth.

96 Hours: Similar to the previous time points, the control group continues to have the highest mean embryo length (513.14). Interestingly, the 2mg group shows a higher mean length (413.00) compared to the 3mg group (418.47), possibly indicating a rebound effect or variability in response.

120 Hours: Once again, the control group demonstrates the highest mean embryo length (524.48), followed by the 2mg (499.58) and 3mg (490.23) groups. The 2mg group displays a notable increase in mean length compared to the 96-hour mark, while the 3mg group remains relatively stable. The results suggest a dose-dependent effect of paracetamol on embryo length, with higher doses potentially leading to reduced growth rates. However, the 3mg group's fluctuating mean lengths at different time points warrant further investigation into potential factors influencing embryonic development under paracetamol exposure.

TABLE 4.1.1: Representing average mean and standard deviation of chick embryo length on *in ovo* Paracetamol exposure at different developmental period

Groups	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	454.51	±14.13	529.22	±95.27	513.00	±60.37	524.48	±41.56
2mg	426.55	±31.78	402.58	±65.82	418.47	±27.21	499.58	±39.95
3mg	401.53	±44.04	523.71	±92.11	413.00	±49.20	490.23	±59.60

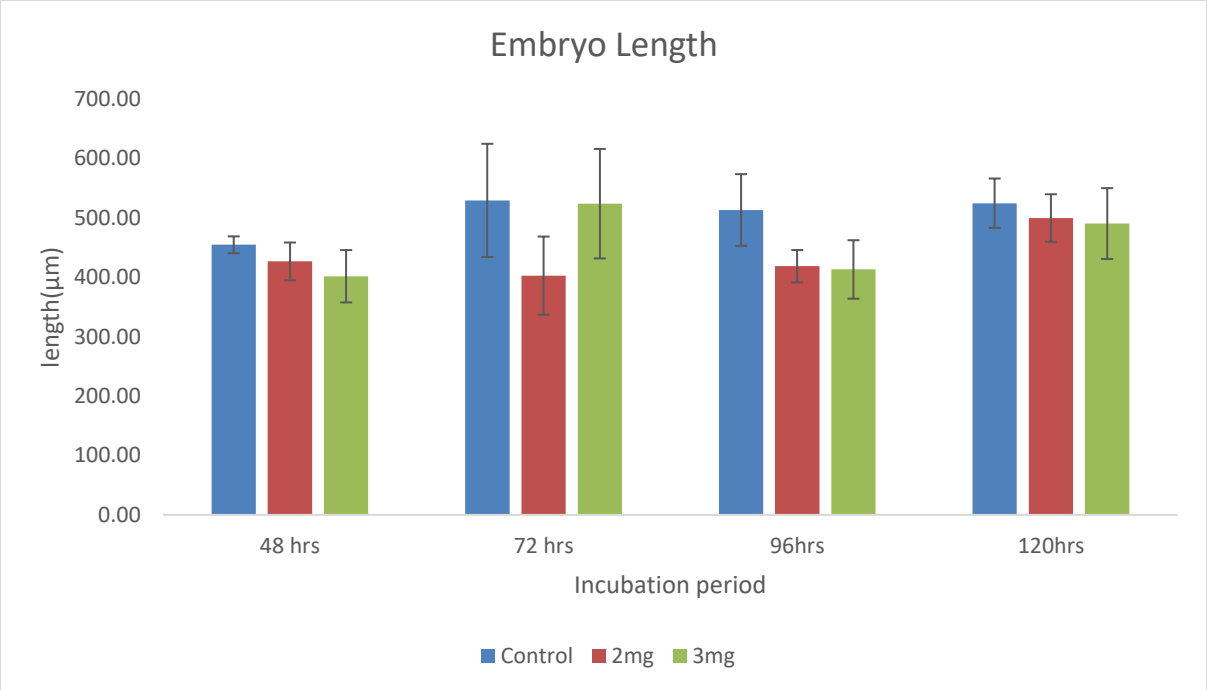

Figure 4.1.1: Graphical representation of average chick embryo length on Paracetamol exposure *in ovo.* Showing decrease in embryo length of Injected groups when compared to Control.

4.1.2 Heart Rate

Table 4.1.2 the mean heart rate of chick embryo at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure.

48 Hours: The control group exhibits a mean heartbeat of 55.17, with a relatively low standard deviation of 6.34. Both the 2mg and 3mg groups show lower mean heartbeats compared to the control group, with values of 48.33 and 52.33, respectively. The standard deviations for these groups are also relatively low, indicating consistency within each group.

72 hours: The mean heartbeat increases across all groups compared to the 48-hour mark. The control group has the highest mean heartbeat at 58.50, followed by the 3mg group at 53.00 and the 2mg group at 45.00. Interestingly, the 2mg group shows a larger standard deviation compared to the other groups, suggesting increased variability in heart rates within this group.

96 Hours: The mean heartbeats continue to increase for all groups. The control group maintains the highest mean heartbeat (63.83), followed by the 3mg group (62.67) and the 2mg group (52.67). The standard deviations remain relatively consistent across all groups, indicating consistent variability in heart rates.

120 Hours: The mean heartbeats decrease slightly for all groups compared to the 96-hour mark. The control group once again exhibits the highest mean heartbeat (54.83), followed by the 3mg group (44.67) and the 2mg group (38.67). The standard deviations remain relatively low for all groups, suggesting consistent variability in heart rates.

The data suggest that paracetamol exposure may have an influence on heartbeat, with fluctuations observed across different dosage groups and time points. However, further analysis and interpretation are required to elucidate the specific effects and underlying mechanisms of paracetamol on embryonic heart development.

TABLE 4.1.2: Representing average mean and standard deviation of heart rate of chick embryo on *in ovo* paracetamol exposure at different developmental period

Groups	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	55.17	±6.34	58.50	±7.64	63.83	±14.29	54.83	±4.02
2mg	48.33	±3.51	45.00	±16.52	52.67	±7.02	38.67	±24.85
3mg	52.33	±4.73	53.00	±14.80	62.67	±11.37	44.67	±6.11

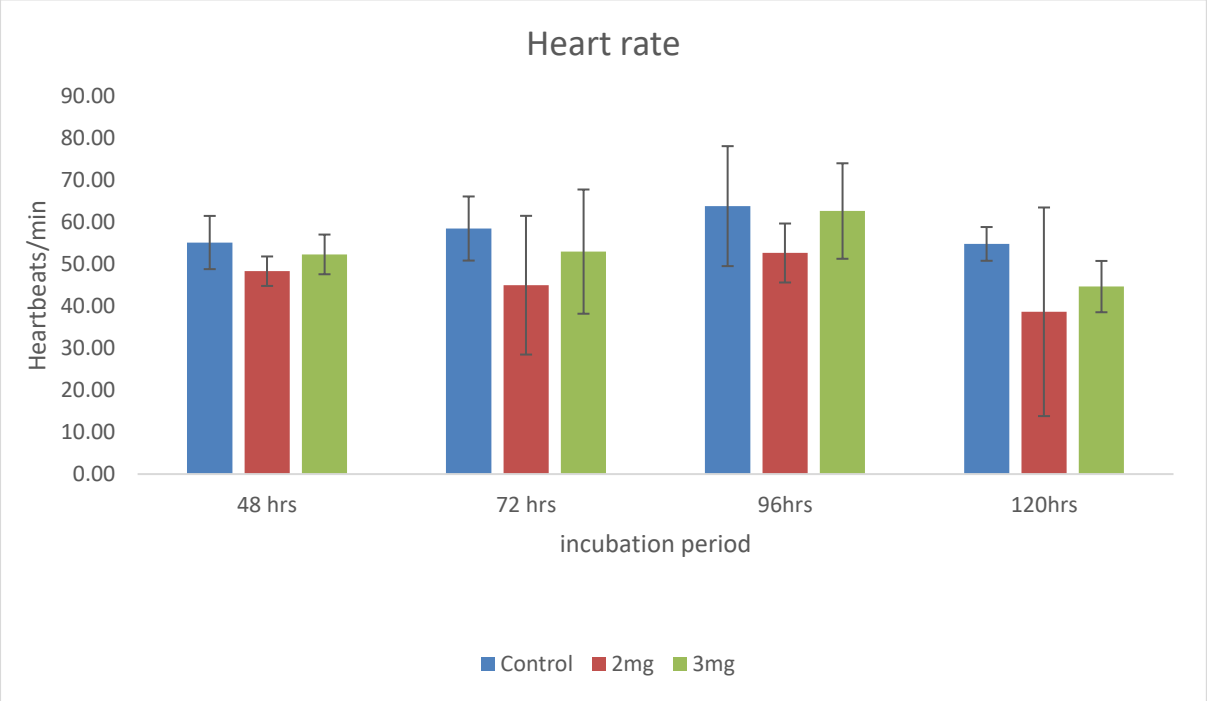

Figure 4.1.2 Average heart rate of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

4.1.3 Anterior Vitelline Vein (AVV):

Table 4.1.3 provides the mean Anterior Vitelline Vein (AVV) at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure.

At 48 hours, the mean diameter of the AVV for the control group is 6.58, with no observed standard deviation (SD), indicating precise measurements. Similarly, for the 2mg and 3mg groups, the mean diameter is 5.38 and 4.83, respectively, with no SD noted. At 72 hours, the mean diameter of the AVV varies across groups, with corresponding SD values indicating the variability in measurements. The trend continues at 96 and 120 hours, with fluctuating mean diameters and corresponding SD values.

4.1.4 Posterior Vitelline Vein (PVV):

Table 4.1.4 provides the mean Posterior Vitelline Vein (PVV) at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure.

Similar to the AVV, the mean diameter of the PVV at 48 hours shows no deviation for all groups, suggesting precise measurements. The mean diameter varies across groups at subsequent time points, with corresponding SD values reflecting the variability in measurements. At 72, 96, and 120 hours, the mean diameter of the PVV fluctuates, with SD values indicating the variability in measurements within each group and time point.

The data demonstrate that the diameter of both the anterior and posterior vitelline veins fluctuates over time and varies between the control and treatment groups. The absence of SD in some measurements indicates precise data collection, while SD values highlight the variability in measurements, which should be considered in the interpretation of results.

TABLE 4.1.3: Representing the mean Anterior Vitelline Vein (AVV) at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure

	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
Groups	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	6.58	±0.45	9.07	±1.43	9.67	±2.07	10.49	±0.33
2mg	5.38	±0.27	6.51	±1.65	8.31	±1.87	9.61	±1.12
3mg	4.83	±0.49	6.20	±1.26	6.50	±2.11	7.02	±1.44

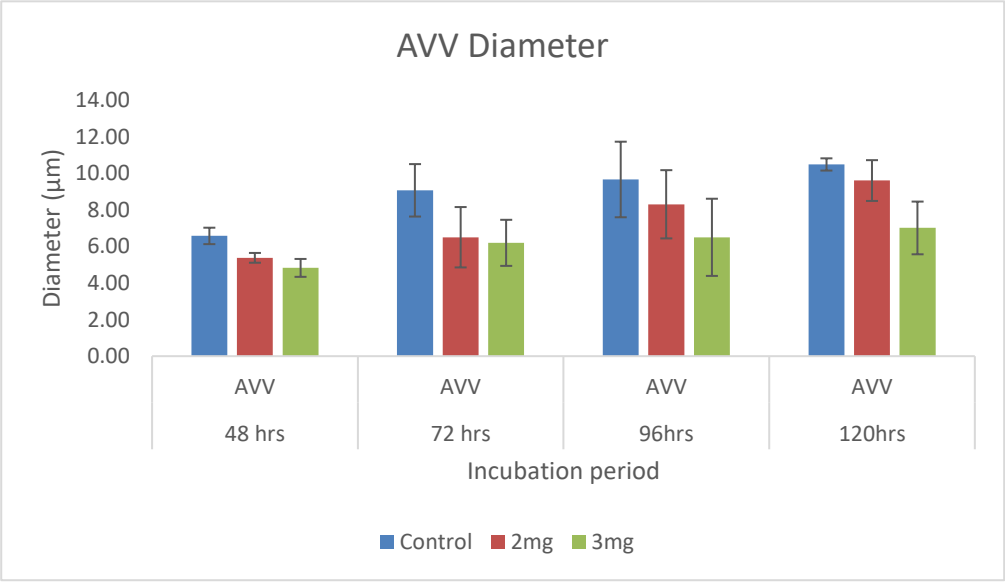

Figure 4.1.3: Average mean AVV diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

TABLE 4.1.4: Representing the mean Posterior Vitelline Vein (PVV) at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure

	Incubation Period					
	72hrs		96hrs		120hrs	
Groups	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	8.90	±2.59	11.11	±2.81	12.17	±0.87
2mg	8.75	±2.62	8.81	±0.90	12.09	±0.46
3mg	8.27	±2.00	8.26	±2.91	8.98	±2.15

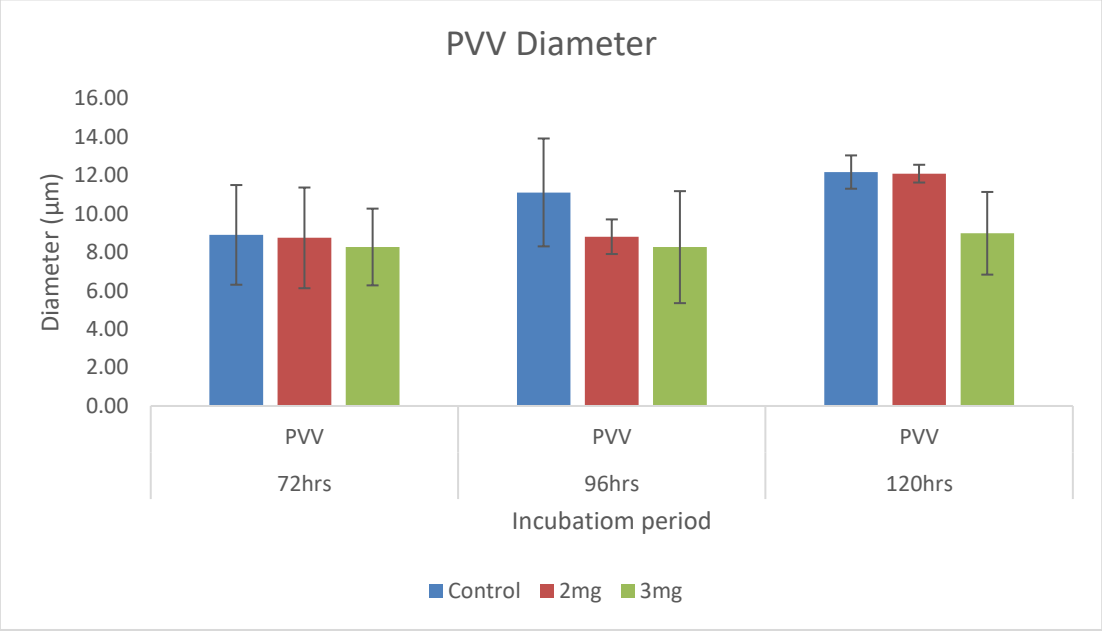

Figure 4.1.4 Average mean PVV diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

4.1.5 Right Vitelline Artery (RVA):

At 48 hours, the mean diameter of the RVA for the control group is 8.23, with a standard deviation (SD) of 0.35. Similarly, for the 2mg and 3mg groups, the mean diameters are 6.23 and 6.90, respectively, with corresponding SD values reflecting variability in measurements (Table 4.1.5).

At 72 hours, the mean diameter of the RVA varies across groups, with corresponding SD values indicating variability in measurements. The trend continues at 96 and 120 hours, with fluctuating mean diameters and corresponding SD values reflecting variability in measurements within each group and time point.

4.1.6 Right Lateral Vitelline Vein (RLVV):

Similar to the RVA, the mean diameter of the RLVV at 48 hours shows variability across groups, with corresponding SD values indicating the variability in measurements (Table 4.1.6).

At subsequent time points (72, 96, and 120 hours), the mean diameter of the RLVV fluctuates, with SD values indicating variability in measurements within each group and time point.

The variability in measurements, as reflected by the standard deviation (SD) values, underscores the importance of considering the consistency and reliability of the data when interpreting the results. It's essential to account for this variability to draw accurate conclusions regarding the effects of the treatments on the diameter of the RVA and RLVV over time.

TABLE 4.1.5: Average mean of measurements of Right vitelline artery (RVA) of chick embryos on *in ovo* paracetamol at different developmental period (48hrs, 72hrs,96hrs & 120hrs)

	Incubation period							
	48hrs		**72hrs**		**96hrs**		**120hrs**	
Groups	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**
Control	8.23	±0.35	11.04	±2.14	12.82	±3.88	13.41	±2.12
2mg	6.23	±0.25	7.67	±2.88	12.85	±2.05	11.04	±2.75
3mg	6.90	±0.47	7.96	±2.93	10.00	±1.99	11.61	±1.67

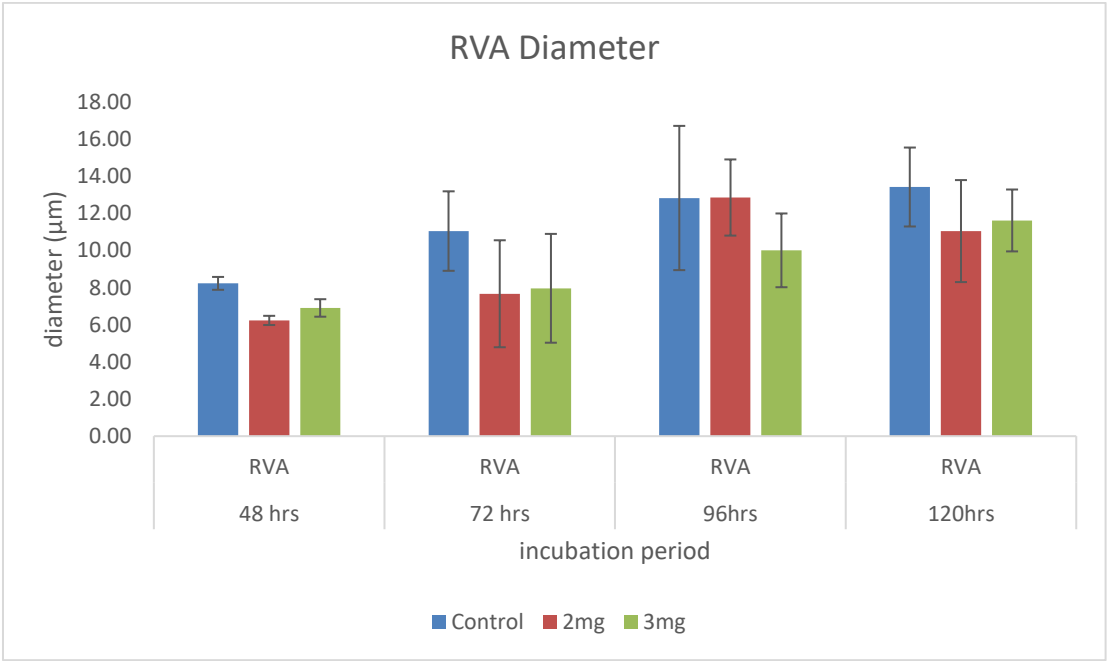

Figure 4.1.5: Average mean RVA diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

TABLE 4.1.6: Average mean of measurements of Right lateral vitelline vein (RLVV) of chick embryos on *in ovo* paracetamol at different incubation period (48hrs, 72hrs,96hrs & 120hrs)

Groups	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	7.05	±0.47	10.20	±1.62	12.03	±1.36	15.97	±0.44
2mg	5.91	±0.32	7.61	±3.19	11.82	±4.94	9.27	±0.60
3mg	6.18	±0.49	7.26	±1.07	10.24	±2.62	8.98	±1.13

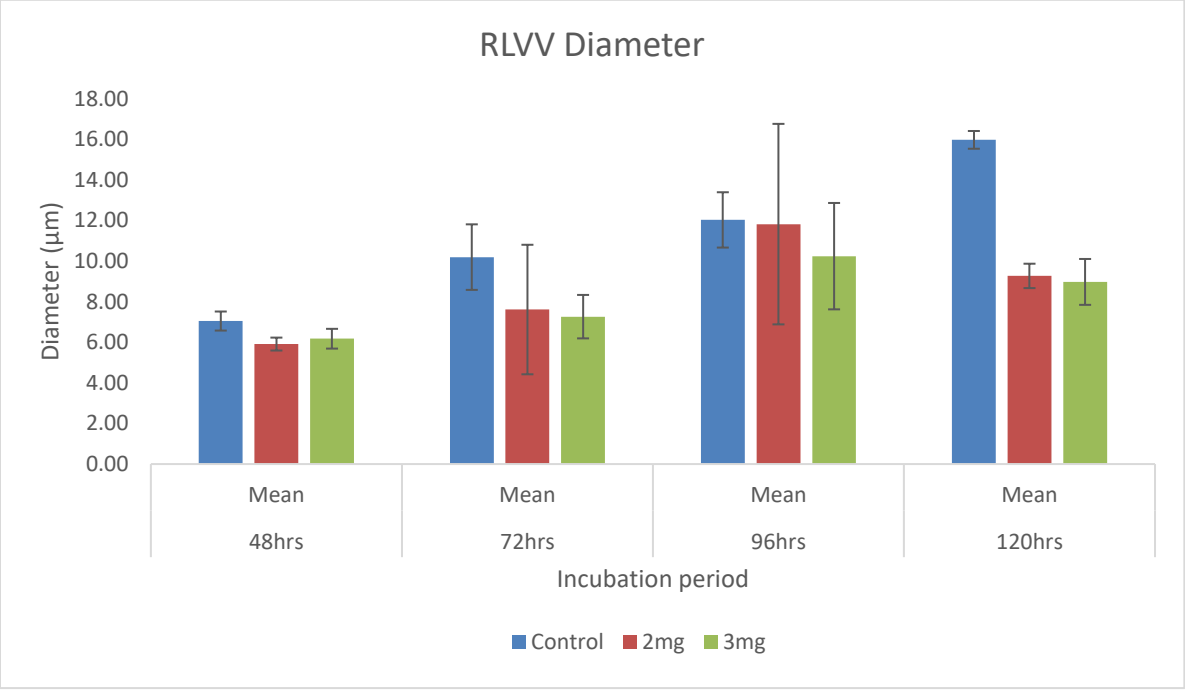

Figure 4.1.6: Average mean RVA diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

4.1.7 Left Vitelline Artery (LVA):

At 48 hours, the mean diameter of the LVA for the control group is 5.83, with a standard deviation (SD) of 0.22. Similarly, for the 2mg and 3mg groups, the mean diameters are 4.60 and 4.95, respectively, with corresponding SD values reflecting variability in measurements (Table 4.1.7).

At 72 hours, the mean diameter of the LVA varies across groups, with corresponding SD values indicating variability in measurements. The trend continues at 96 and 120 hours, with fluctuating mean diameters and corresponding SD values reflecting variability in measurements within each group and time point.

4.1.8 Left Lateral Vitelline Vein (LLVV):

Similar to the LVA, the mean diameter of the LLVV at 48 hours shows variability across groups, with corresponding SD values indicating the variability in measurements (Table 4.1.8).

At subsequent time points (72, 96, and 120 hours), the mean diameter of the LLVV fluctuates, with SD values indicating variability in measurements within each group and time point.

The variability in measurements, as reflected by the standard deviation (SD) values, underscores the importance of considering the consistency and reliability of the data when interpreting the results. It's essential to account for this variability to draw accurate conclusions regarding the effects of the treatments on the diameter of the LVA and LLVV over time.

TABLE 4.1.7: Representing the average mean of Left Vitelline Artery (LVA) diameter at different time points across three groups: Control, 2mg, and 3mg paracetamol exposure

Groups	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	5.83	±0.22	7.47	±0.75	10.86	±1.68	14.29	±2.08
2mg	4.60	±0.14	5.15	±1.43	9.86	±4.26	8.15	±0.54
3mg	4.95	±0.46	6.35	±1.14	7.68	±3.09	12.13	±3.98

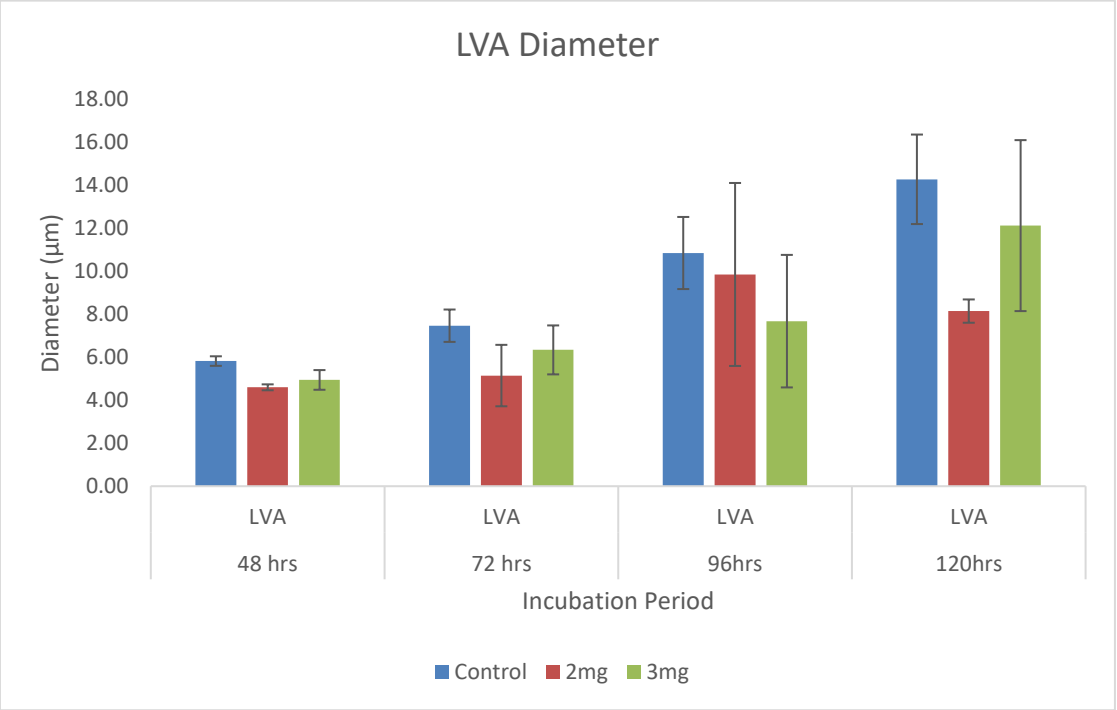

Figure 4.1.7: Average mean LVA diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

TABLE 4.1.8: Representing average mean and standard deviation of Left Lateral Vitelline Vein (LLVV) diameter on *in ovo* paracetamol exposure at different incubation period

Groups	Incubation period							
	48hrs		72hrs		96hrs		120hrs	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Control	12.31	±1.16	8.36	±1.32	13.02	±2.51	11.94	±1.40
2mg	8.03	±0.51	5.85	±1.77	12.19	±1.92	11.69	±2.27
3mg	8.12	±1.00	6.31	±1.10	8.78	±3.05	10.88	±0.99

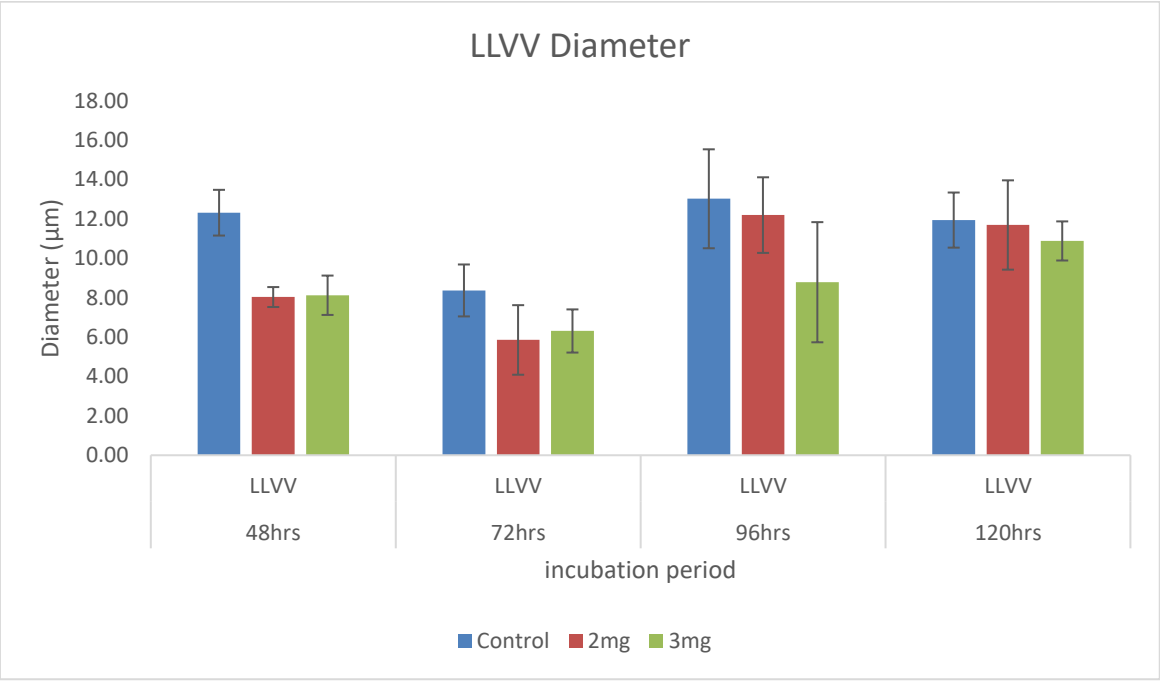

Figure 4.1.8: Average mean LLVV diameter of chick embryos injected with Paracetamol (2mg, 3mg) and control at (48hrs, 72hrs, 96hrs and 120 hrs of incubation)

During *in ovo* Paracetamol exposure, the crown to rump length measurements of chick embryos at 48, 72, 96, and 120 hours of incubation did not show prominent differences following injection with 2mg and 3mg of Paracetamol per egg. This indicates that Paracetamol administration at these dosage levels did not notably impact the growth and development of chick embryos during the specified incubation periods.

Heart Rate Response to Paracetamol Exposure Paracetamol administration showed no significant impact on the heartbeat of chick embryos. Embryos injected with either 2mg or 3mg of paracetamol did not exhibit a notable difference in heartbeat compared to the control group during the 2-5day incubation period. These results indicate that paracetamol injection at these doses did not significantly affect embryonic cardiac activity in early development.

Vitelline Vessel Diameter Response to Paracetamol Exposure: In 48-hour chick embryos, changes in vessel diameter were primarily observed in the Right Lateral Vitelline Vein following injections of both 2mg and 3mg of paracetamol. However, other vessels such as the Anterior Vitelline Vein, Right Vitelline Artery, Left Vitelline Artery, and Left Lateral Vitelline Vein did not show much alterations in diameter in response to paracetamol administration. By the 72-hour mark, the Right Lateral Vitelline Vein displayed a visual difference in diameter with injections of 3mg paracetamol, while other vessels remained unaffected. At 96 hours, only the Posterior Vitelline Vein exhibited a variation in diameter when administered 2mg of paracetamol. Conversely, no specific differences were observed in the diameter of other vitelline vessels at this stage. By the 120-hour mark, alterations in vessel diameter were noted, particularly in the Right Lateral Vitelline Vein with injections of both 2mg and 3mg of paracetamol. Additionally, the diameter of the Left Vitelline Artery showed prominent differences following administration of 2mg of paracetamol. However, no changes were detected in the remaining vessels assessed at this developmental stage. These results suggest that paracetamol administration can lead to significant alterations in vitelline vessel diameter,

particularly in specific vessels at different developmental stages. However, the overall impact of paracetamol on vitelline vessel morphology appears to vary depending on the developmental stage. Further investigation is needed to understand the underlying mechanisms and implications of these observed alterations for embryonic development.

At 48 Hours of Development: In the control group, vitelline vessels formed a network of capillaries on the yolk's surface, facilitating nutrient absorption and transportation to the embryo. These vessels were concentrated within specific blood vessels in the yolk sac, particularly in the sinus terminalis, extending to the anterior, posterior, right, and left lateral vitelline veins. However, embryos injected with 2mg and 3mg of paracetamol exhibited a similar arrangement but with fainter circulation, particularly in smaller vessels.

At 96 Hours of Development: In the control group, vitelline vessels were uniformly spread across the sinus terminalis, displaying distinct bifurcations and pigmentation. However, minor alterations were observed in the experimental group administered 2mg and 3mg of paracetamol, characterized by reduced circulation and slightly diminished pigmentation. Additionally, a notable bifurcation near the origins of the right and left vitelline arteries was identified.

At 120 Hours of Development: The observed vitelline vessels displayed a well-organized arrangement with uniform distribution, predominantly in the area pellucida. Clear evidence of proper bifurcations indicated a structured branching pattern essential for efficient nutrient and fluid exchange. Moreover, vessels exhibited distinct pigmentation, suggesting functional maturity. However, in experimental embryos treated with 2mg and 3mg of paracetamol, no significant alterations in vitelline vessel morphology were observed.

These findings suggest that while paracetamol administration may induce subtle changes in vitelline vessel characteristics during early developmental stages, its impact appears to diminish by later stages.

***In Vitro* Paracetamol Exposure**: Control Group: In the control group, the area vasculosa region of chick embryos at 48, 72, 96, and 120 hours displayed a robust vascular network, including the anterior and posterior vitelline veins, as well as the left and right omphalomesenteric vessels. A dense capillary network was evident at both the proximal and distal ends of the area vasculosa. As developmental stages progressed, there was a noticeable increase in the radius of the area opaca, indicating ongoing vascular expansion and morphological changes essential for nutrient exchange and embryonic growth.

After Exposure to Paracetamol (*In Vitro*): Upon exposure to approximately 3mg of paracetamol, administered via Whatman No.1 filter paper applied to different vitelline vessels, distinct morphological alterations were observed at various developmental stages of chick embryos. At 48 hours post-exposure, a pronounced fading of major vessels was noted at both proximal and distal ends. Subsequent observations at 72 hours revealed regression of the capillary network from both proximal and distal regions of the area vasculosa. Remarkably, embryos at 96 and120 hours post-exposure exhibited fading of capillaries, while major vessels remained relatively unaffected.

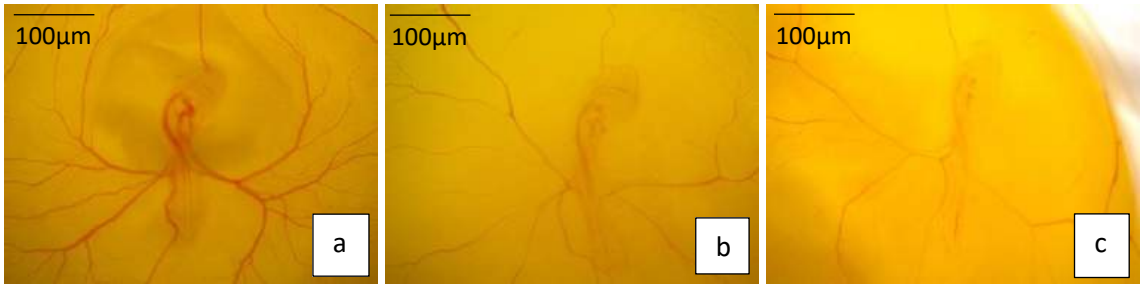

Figure 4.1.8: Chick embryos exposed to Paracetamol (*in ovo*) at 72hrs of incubation; a) Control, b) 2mg paracetamol and c) 3mg Paracetamol

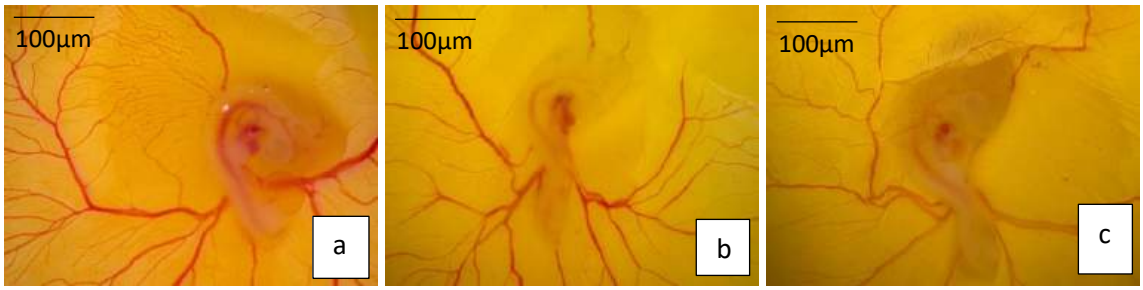

Figure 4.1.9: Chick embryos exposed to Paracetamol (*in ovo*) at 96hrs of incubation; a) Control, b) 2mg paracetamol and c) 3mg Paracetamolv

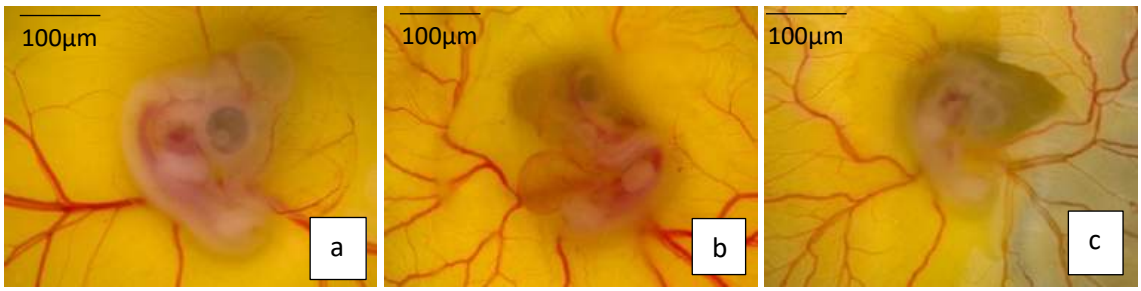

Figure 4.2.0: Chick embryos exposed to Paracetamol (*in ovo*) at 120hrs of incubation; a) Control, b) 2mg paracetamol and c) 3mg Paracetamol

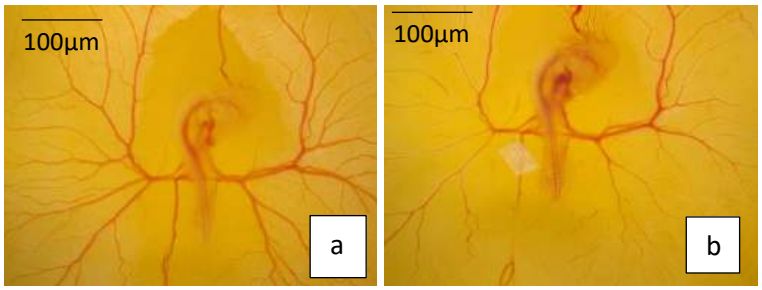

Figure 4.2.1: *in vitro* exposure of Paracetamol to chick embryos at 72hrs of incubation;
a) before exposure, b) after exposure

4.2 Discussion

The analysis of embryo length at various developmental time points and across different dosage groups revealed a clear pattern indicating a dose-dependent effect of paracetamol on embryonic growth. Specifically, embryos exposed to higher doses of paracetamol exhibited reduced growth rates compared to those in the control group or exposed to lower doses. This consistent trend across multiple time points strongly suggests that paracetamol has an inhibitory effect on embryonic growth, with higher doses resulting in more pronounced growth suppression.

However, an intriguing aspect emerged from the data, particularly in the 3mg dosage group, where mean embryo lengths fluctuated at different time points. While overall, the trend of reduced growth rates with increasing dosage remained evident, the variability in mean lengths within the 3mg group raises questions about the consistency of the response to paracetamol exposure. This variability suggests that factors beyond dosage alone may influence embryonic development under paracetamol exposure.

The observed fluctuation in mean embryo lengths within the 3mg group underscores the complexity of the relationship between paracetamol exposure and embryonic growth. It implies that other variables, such as individual embryo susceptibility, developmental stage-specific responses, or potential compensatory mechanisms, may contribute to the variability in growth outcomes. Therefore, further investigation is warranted to explore these factors and elucidate the underlying mechanisms driving the observed variability in embryonic development under paracetamol exposure.

Understanding the factors influencing embryonic growth under paracetamol exposure is crucial for assessing the safety and potential risks associated with its use during pregnancy. By

unravelling the complexities of the dose-response relationship and variability in growth outcomes, future research can provide valuable insights into the precise mechanisms underlying paracetamol-induced developmental abnormalities and inform strategies to mitigate potential adverse effects on fetal health and well-being.

Secondly, the assessment of heart rate across dosage groups and various time points highlighted fluctuations, indicating a potential influence of paracetamol exposure on embryonic cardiac activity. This observation aligns with prior studies implicating paracetamol in cardiovascular alterations during embryonic development. While the precise mechanisms driving these fluctuations remain unclear, the observed changes in heart rate serve as crucial indicators of potential cardiac disturbances induced by paracetamol. It's essential to delve deeper into these fluctuations to understand whether they result from direct drug effects on cardiac tissue or secondary effects mediated through other physiological pathways. Further investigation into the molecular and cellular mechanisms underlying these alterations could provide valuable insights into the cardiotoxic effects of paracetamol and inform clinical risk assessments associated with its use during pregnancy.

Additionally, the examination of vitelline vessel diameter in response to paracetamol exposure unveiled notable alterations, particularly affecting specific vessels at different developmental stages. These findings indicate that paracetamol administration can induce disruptions in vitelline circulation, potentially interfering with crucial processes such as nutrient exchange and embryonic development. This aligns with existing literature suggesting that paracetamol can perturb vascular function and morphology during embryogenesis. However, the variability observed in vessel diameter changes across various time points and dosage groups underscores the multifaceted nature of paracetamol's effects on embryonic vascular morphology. These variations may stem from complex interactions between paracetamol and developmental processes, necessitating further investigation into the underlying mechanisms driving these

alterations. Understanding the intricacies of paracetamol-induced changes in vascular morphology is crucial for delineating its potential teratogenic effects and informing clinical decisions regarding its use in pregnant individuals.

The selection of vitelline circulation parameters, including the diameter of vitelline vessels such as the anterior and posterior vitelline veins, as well as the left and right vitelline arteries, was a judicious choice for this study. These parameters play crucial roles in embryonic development and nutrient exchange. The vitelline circulation system acts as a lifeline for the developing embryo, facilitating the transfer of essential nutrients, oxygen, and metabolic waste products between the yolk sac and the embryo. Any disruptions in this intricate network could profoundly impact embryonic growth and development. By monitoring changes in vitelline vessel diameter, researchers could gain valuable insights into the potential mechanisms underlying paracetamol-induced developmental abnormalities. Alterations in vessel diameter may indicate disturbances in blood flow dynamics, nutrient transport efficiency, or vascular development, shedding light on how paracetamol exposure affects embryonic vascular morphology and function.

Additionally, the assessment of heart rate served as a vital indicator of cardiovascular function and overall embryonic health. The embryonic heart is among the first organs to develop and plays a fundamental role in supplying oxygenated blood to tissues and organs throughout the embryo. Changes in heart rate can reflect alterations in cardiac activity, circulation, and cardiovascular function. By monitoring heart rate, researchers could evaluate the immediate effects of paracetamol exposure on embryonic cardiac dynamics. Fluctuations in heart rate may signify disruptions in cardiac function or cardiac developmental abnormalities induced by paracetamol, providing crucial insights into the drug's impact on embryonic circulatory dynamics and cardiovascular health. In totality, the inclusion of vitelline circulation parameters and heart rate assessment in the study design enhanced our understanding of paracetamol's

effects on embryonic development and cardiovascular function, contributing to the broader knowledge of the drug's safety profile during pregnancy.

4.3 Conclusions

Based on the study conducted by the author, several conclusions can be drawn:

Paracetamol Dosage and Embryonic Growth: The research indicates a dose-dependent effect of paracetamol on embryonic growth, with higher doses correlating with reduced growth rates. This suggests that caution should be exercised regarding the dosage of paracetamol administered during pregnancy to minimize potential adverse effects on embryonic development.

Cardiac Activity and Paracetamol Exposure: Fluctuations observed in heart rate across dosage groups and time points suggest a potential influence of paracetamol exposure on embryonic cardiac activity. While the exact mechanisms remain unclear, these findings underscore the need for further investigation into the effects of paracetamol on cardiac function during early development.

Vitelline Vessel Morphology and Paracetamol Administration: Significant alterations in vitelline vessel diameter indicate disruptions in vitelline circulation in response to paracetamol exposure. This disruption may impact nutrient exchange and embryonic development. However, the variability observed across time points and dosage groups highlights the complexity of paracetamol's effects on embryonic vascular morphology.

Implications for Clinical Practice: The study's findings have implications for clinical practice and public health policies regarding the use of paracetamol during pregnancy. Healthcare providers should consider the potential risks associated with paracetamol exposure and weigh them against the benefits when prescribing this medication to pregnant individuals.

Hence, the study provides valuable insights into the effects of paracetamol exposure on embryonic development. By elucidating these effects, the research contributes to a better understanding of the safety profile of paracetamol during pregnancy and informs clinical

decision-making to promote maternal and fetal health. However, further research is needed to fully understand the mechanisms underlying paracetamol-induced developmental abnormalities and to develop strategies to mitigate potential risks associated with its use during pregnancy.

References

- Anderson-Berry, A., O'Brien, E. A., Bleyl, S. B., Lawson, A., Gundersen, N., Ryssman, D., … Albertine, K. H. (2005). Vasculogenesis drives pulmonary vascular growth in the developing chick embryo. *Developmental Dynamics*, 233(1), 145–153.
- Bauer, A. Z., Swan, S. H., Kriebel, D., Liew, Z., Taylor, H. S., Bornehag, C. G., ... & Kristensen, D. M. (2021). Paracetamol use during pregnancy—a call for precautionary action. *Nature Reviews Endocrinology*, 17(12), 757-766.
- Blecharz-Klin, K., Joniec-Maciejak, I., Jawna, K., Pyrzanowska, J., Piechal, A., Wawer, A., & Widy-Tyszkiewicz, E. (2015). *Developmental exposure to paracetamol causes biochemical alterations in medulla oblongata. Environmental Toxicology and Pharmacology, 40(2), 369–374.*
- Blecharz-Klin, K., Piechal, A., Jawna-Zboińska, K., Pyrzanowska, J., Wawer, A., Joniec-Maciejak, I., & Widy-Tyszkiewicz, E. (2017). Paracetamol− Effect of early exposure on neurotransmission, spatial memory and motor performance in rats. *Behavioural Brain Research*, *323*, 162-171.
- Bremer, L., Goletzke, J., Wiessner, C., Pagenkemper, M., Gehbauer, C., Becher, H., … Tiegs, G. (2017). Paracetamol Medication During Pregnancy: Insights on Intake Frequencies, Dosages and Effects on Hematopoietic Stem Cell Populations in Cord Blood From a Longitudinal Prospective Pregnancy Cohort. *EBioMedicine*, 26, 146–151.
- Burdan, F. (2004). Developmental effects of propyphenazone in analgesic and antipyretic combination with caffeine or paracetamol. *Human & Experimental Toxicology*, 23(5), 235–244.
- Burggren, W. W. (2013). Cardiovascular development and angiogenesis in the early vertebrate embryo. *Cardiovascular engineering and technology*, 4, 234-245.

- Callebaut, M., Van Nueten, E., Bortier, H., & Harrisson, F. (2003). Induction of the avian coelom with associated vitelline blood circulation by Rauber's sickle derived junctional endoblast and its fundamental role in heart formation. *Journal of Morphology*, 259(1), 21–32.
- Chumpanya, N., Plakornkul, V., Roongruangchai, J., Viravud, Y., & Rungruang, T. (2020, August). The Teratogenic Effects of Ibuprofen on Developing in Ovo Chick Embryo. In *Rangsit Graduate Research Conference: RGRC* (Vol. 15, No. 2563), pp. 2737-2743).
- David, A., & Pancharatna, K. (2009). Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio. *Journal of applied toxicology*, 29(7), 597-602.
- De Melo Bernardo, A., Sprenkels, K., Rodrigues, G., Noce, T., & Chuva De Sousa Lopes, S. M. (2012). Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation. *Biology open*, 1(11), 1146-1152.
- ElMazoudy, R. H., & Bekhet, G. A. (2016). In ovo toxico-teratological effects of aluminum on embryonic chick heart and vascularization. *Environmental Science and Pollution Research*, 23, 21947-21956.
- Fernandes, N. V. (2017). Evaluation of genotoxic, embryotoxic and teratogenic potential of paracetamol in humans and mice. *Journal of Medical Science and Clinical Research*, *05*(02), 17324–17329.
- Gonzalez-Crussi, F. (1971). Vasculogenesis in the chick embryo. An ultrastructural study. American Journal of Anatomy, 130(4), 441-459.
- Graham, G. G., Davies, M. J., Day, R. O., Mohamudally, A., & Scott, K. F. (2013). The modern pharmacology of paracetamol: therapeutic actions, mechanism of action,

metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology*, 21, 201-232.

- Haider ali naser. (2022). Development of the circulatory system in the chicken embryo. *Eurasian Medical Research Periodical*, *9*, 10–20.
- Hamburger V. Hamilton HL. A series of normal stages in the development of chick embryo. J Morphol 1951; 88: 49-92.
- Hogers, B., DeRuiter, M. C., Baasten, A. M. J., Gittenberger-de Groot, A. C., & Poelmann, R. E. (1995). Intracardiac blood flow patterns related to the yolk sac circulation of the chick embryo. *Circulation research*, 76(5), 871-877.
- Hogers, B., DeRuiter, M. C., Baasten, A. M. J., Gittenberger-de Groot, A. C., & Poelmann, R. E. (1995). Intracardiac blood flow patterns related to the yolk sac circulation of the chick embryo. *Circulation research*, 76(5), 871-877.

  Hu, N., & Clark, E. B. (1989). Hemodynamics of the stage 12 to stage 29 chick embryo. Circulation Research, 65(6), 1665–1670.
- Hu, N., Ngo, T. D., & Clark, E. B. (1996). Distribution of blood flow between embryo and vitelline bed in the stage 18, 21 and 24 chick embryo. *Cardiovascular research*, 31(supp1), E127-E131.
- Kabir, A. (2012). Haematological studies in chicken and a group of birds. *International Journal of Medical and Applied Sciences*, *1*(1), 30-38.
- Kain, K. H., Miller, J. W., Jones-Paris, C. R., Thomason, R. T., Lewis, J. D., Bader, D. M., ... & Zijlstra, A. (2014). The chick embryo as an expanding experimental model for cancer and cardiovascular research. *Developmental Dynamics*, *243*(2), 216-228.
- Khosravi, A., Sharifi, I., Tavakkoli, H., Derakhshanfar, A., Keyhani, A. R., Salari, Z., ... & Bamorovat, M. (2018). Embryonic toxico-pathological effects of meglumine antimoniate using a chick embryo model. *PloS one*, 13(5), e0196424.

- Kilcoyne, K. R., & Mitchell, R. T. (2017). Assessing the impact of in-utero exposures: potential effects of paracetamol on male reproductive development. *Archives of Disease in Childhood*, 102(12), 1169–1175.
- Kotwani, A. (1998). Use of chick embryo in screening for teratogenicity. *Indian journal of physiology and pharmacology*, *42*, 189-204.
- Labba, N. A., Wæhler, H. A., Houdaifi, N., Zosen, D., Haugen, F., Paulsen, R. E., ... & Eskeland, R. (2022). Paracetamol perturbs neuronal arborization and disrupts the cytoskeletal proteins SPTBN1 and TUBB3 in both human and chicken in vitro models. *Toxicology and Applied Pharmacology*, 449, 116130.
- Lis, M. W., Sechman, A., Niedziolka, J. W., & Rzasa, J. (2006). Effect of paracetamol injection in ovo in the course of hatching and thyroid hormone levels in chicken embryos. BULLETIN-VETERINARY INSTITUTE IN PULAWY, 50(4), 537.
- Mobbs, I. G., & McMillan, D. B. (1979). Structure of the endodermal epithelium of the chick yolk sac during early stages of development. *American Journal of Anatomy*, 155(3), 287-309.
- Moungmaithong, S., Leung, B. W., Sahota, D. S., Wang, C. C., Leung, T. Y., & Poon, L. C. (2022). Assessment of embryo morphology following perinatal exposure to aspirin, ibuprofen and paracetamol using whole embryo culture system. *The Journal of Maternal-Fetal & Neonatal Medicine*, 35(25), 8786-8793.
- Murphy, M. E., & Carlson, E. C. (1978). An ultrastructural study of developing extracellular matrix in vitelline blood vessels of the early chick embryo. *American Journal of Anatomy*, 151(3), 345–375.
- Siamwala, J. H., Dias, P. M., Majumder, S., Joshi, M. K., Sinkar, V. P., Banerjee, G., & Chatterjee, S. (2013). L-theanine promotes nitric oxide production in endothelial cells

through eNOS phosphorylation. *The Journal of nutritional biochemistry*, *24*(3), 595-605.

- Swaminathan, A., Balaguru, U. M., Manjunathan, R., Bhuvaneswari, S., Kasiviswanathan, D., Sirishakalyani, B., ... & Chatterjee, S. (2019). Live Imaging and Analysis of Vasoactive Properties of Drugs Using an in-ovo Chicken Embryo Model: Replacing and Reducing Animal Testing. *Microscopy and Microanalysis*, *25*(4), 961-970.
- Tufan, A. C., Abban, G., Akdogan, I., Erdogan, D., & Ozogul, C. (2007). The effect of in ovo ethanol exposure on retina and optic nerve in a chick embryo model system. *Reproductive Toxicology*, *23*(1), 75-82.
- Vergara, M. N., & Canto-Soler, M. V. (2012). Rediscovering the chick embryo as a model to study retinal development. *Neural development*, *7*, 1-19.