Assessment of the Combined Influence of Caffeine and Aspartame on Mouse Ovary

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Assessment of the Combined Influence of Caffeine and Aspartame on Mouse Ovary"**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 / Mentorship of Dr. Shanti N. Dessai and the same has not been submitted elsewhere for the award of a degreeor 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 in the dissertation.

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This is to certify that the dissertation report **"Assessment of the Combined Influence of Caffeine and Aspartame on Mouse Ovary"** is a bonafide work carried out by **Ms. Racheal Renny D'souza** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science in Zoology** in the Zoology Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

This thesis is submitted in fulfilment of the requirement for the degree of Master's in Zoology and comprises research work carried out by the author under the guidance of Dr. Shanti N. Dessai, Assistant Professor, Zoology Discipline, Goa University from 2023 to 2024.

The research presented in this dissertation explores the combined effects of aspartame and caffeine on the female reproductive parameters of mice. This investigation emerged from a growing interest in understanding the potential impacts of commonly consumed food additives in beverages and other products on mammalian physiology, particularly within the context of female reproductive health. Aspartame, an artificial sweetener, and caffeine, a widely consumed stimulant, are frequently encountered in modern diet in combination. Despite their prevalent use, their effects on reproductive function, especially in combination, remain relatively understudied. Thus, investigations of the combined effects of caffeine and aspartame on the female reproductive parameters of *Mus musculus* will provide data about its reproductive toxicity that can be further used to generate preventive measures to reduce the exposure to caffeine and aspartame in combination.

The primary objectives of this study are to evaluate the changes in body weight, reproductive organ weight, GSI, estrous cycle lengths and histology of ovaries.

The thesis is divided into five main chapters. The first chapter, introduction, gives an overview about aspartame and caffeine, their use in modern diet, mechanism of action in the body after consumption and the aims and objectives of the project. The second chapter includes the survey of literature. Chapter 3 gives the materials and methods used for the study. Chapter 4 represents the results embodying observations of changes in body weight, GSI, estrous cycle and histology of ovaries. Chapter 5 gives elaborate discussions about the reasons and effects of changes occurring in the female reproductive parameters mice as a result of caffeine and aspartame toxicity.

Undertaking this research was both challenging and rewarding. I am deeply grateful to my dissertation committee and academic advisors for their invaluable guidance and support throughout this journey. Their expertise and encouragement have been instrumental in shaping the direction and rigor of this investigations.

CHAPTER 1: INTRODUCTION

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1.1 Background

The contemporary lifestyle is marked by a substantial rise in the consumption of artificial sweeteners and caffeinated beverages, both of which are prevalent in various food and beverage products. Among these, aspartame, a low-calorie sweetener, and caffeine, a natural stimulant, have garnered considerable attention due to their ubiquitous presence in modern diets. The increasing prevalence of artificial sweeteners and caffeinated beverages in contemporary diets has prompted extensive research into their individual health effects (Smith *et al*., 2019; Brown & Jones, 2020). Despite their ubiquity, the potential synergistic effects of these compounds on mammalian physiology, particularly on female reproductive health, remain relatively understudied. This study aims to fill this gap by investigating the combined influence of caffeine and aspartame on the mouse ovary. Understanding the repercussions of caffeine and aspartame interaction on the female reproductive system is of paramount importance due to its potential implications for human health. Aspartame, a commonly used artificial sweetener, and caffeine, a widely consumed stimulant, both exert significant effects on various physiological processes within the body. However, their combined impact on reproductive parameters, particularly on ovarian health, remains a subject of scientific inquiry. Given the increasing consumption of caffeinated beverages and foods containing artificial sweeteners, it is crucial to comprehend the potential consequences of this combined exposure, especially for vulnerable populations such as women of reproductive age. By elucidating the interplay between caffeine and aspartame on the mouse ovary, this research not only contributes to our understanding of basic reproductive biology but also sheds light on the potential reproductive toxicity of these commonly consumed compounds.

The present study aims to assess the combined influence of caffeine and aspartame on mouse ovary, investigating their potential synergistic effects on female reproductive parameters. Through comprehensive evaluation of changes in ovarian histology, estrous cycle lengths, body weight, and reproductive organ weight, this research seeks to elucidate the impact of caffeine

and aspartame interaction on ovarian health. By addressing this understudied aspect of dietary influence on reproductive physiology, this study aims to contribute to a better understanding of the potential reproductive toxicity of commonly consumed food additives, informing public health initiatives aimed at promoting reproductive well-being.

1.2 Statement of the study

The present study aims to assess the combined influence of caffeine and aspartame on mouse ovary, investigating their potential synergistic effects on female reproductive parameters. Through comprehensive evaluation of changes in ovarian histology, estrous cycle lengths, body weight, and reproductive organ weight, this research seeks to elucidate the impact of caffeine and aspartame interaction on ovarian health. By addressing this understudied aspect of dietary influence on reproductive physiology, this study aims to contribute to a better understanding of the potential reproductive toxicity of commonly consumed food additives, informing public health initiatives aimed at promoting reproductive well-being.

1.3 Aim and Objectives of the study

The aim is to assess how caffeine and aspartame together affect female reproductive parameters, focusing on the mouse ovary (Through analyzing changes in ovarian histology, estrous cycles, body weight, and reproductive organ weight). Given below are the 2 objectives for this study:

1. To determine changes in estrous cycle lengths, body weight, and reproductive organ weight in response to simultaneous consumption of caffeine and aspartame.
2. To investigate alterations in ovarian histology resulting from combined exposure to caffeine and aspartame in mice.

1.4 Hypotheses of the study

Hypotheses of this study propose that exposure to a combination of caffeine and aspartame will lead to histopathological changes in ovarian tissues and alterations in the length of the estrous cycle compared to the control group. There will also be a significant change in body weight and reproductive organ weight of mice exposed to caffeine and aspartame.

1.5 Research Questions for this study

1. How does combined exposure to caffeine and aspartame affect the histological characteristics of the mouse ovary?
2. What alterations, if any, occur in the estrous cycle lengths of mice following simultaneous consumption of caffeine and aspartame?
3. Are there significant changes in body weight observed in mice exposed to both caffeine and aspartame compared to control groups?
4. How does combined exposure to caffeine and aspartame impact the relative weights of reproductive organs in mice?
5. What are the potential mechanisms underlying any observed alterations in female reproductive parameters following concurrent consumption of caffeine and aspartame?
6. Are there dose-dependent effects of caffeine and aspartame on mouse ovary, and if so, what are the thresholds for observable changes in reproductive parameters?
7. Do any observed effects on female reproductive parameters persist after cessation of caffeine and aspartame exposure, indicating potential long-term reproductive toxicity?
8. How do the findings of this study contribute to our understanding of the reproductive health implications associated with the combined consumption of caffeine and aspartame, and what implications do they hold for human health?

1.6 Possible solutions based on this study

Based on the findings of this study, several potential solutions or recommendations can be proposed:

Public Awareness and Education: Increase public awareness regarding the potential combined effects of caffeine and aspartame on reproductive health through educational campaigns, aiming to inform individuals, especially women of reproductive age, about the potential risks associated with excessive consumption of caffeinated beverages and products containing artificial sweeteners.

Regulatory Measures: Advocate for stricter regulations and labelling requirements for products containing caffeine and aspartame, ensuring consumers are informed about the presence and potential health effects of these additives. This could include clearer labelling of caffeine and aspartame content on food and beverage packaging, as well as restrictions on their use in certain products, particularly those marketed towards pregnant women or individuals trying to conceive.

Further Research: Encourage further research into the long-term effects of combined caffeine and aspartame exposure on reproductive health, including studies examining potential transgenerational effects and the underlying molecular mechanisms involved. This could help to better understand the full extent of reproductive toxicity associated with these compounds and inform the development of targeted interventions and treatments.

Alternative Sweeteners and Beverages: Promote the consumption of beverages and foods sweetened with alternative, naturally-derived sweeteners that have been shown to be safe for reproductive health. This could include options such as stevia, erythritol, or monk fruit extract, which may provide viable alternatives to aspartame in certain products.

Individual Dietary Choices: Encourage individuals to make informed dietary choices and limit their consumption of caffeinated beverages and products containing artificial sweeteners,

particularly if they are trying to conceive or are concerned about their reproductive health. Encouraging a balanced diet rich in whole foods and low in processed products can also help to minimize exposure to potentially harmful additives.

1.7 Scope of the study

The scope of this study involves a detailed investigation into the combined effects of caffeine and aspartame on female reproductive parameters, specifically focusing on the mouse ovary. It includes analyzing changes in ovarian histology, estrous cycle lengths, body weight, and reproductive organ weight following concurrent exposure to caffeine and aspartame.

Through controlled experimentation and meticulous data analysis, the study aims to uncover potential synergistic effects on reproductive health, contributing to a deeper understanding of the reproductive toxicity associated with these widely consumed food additives. Additionally, the study seeks to explore underlying mechanisms driving any observed alterations in reproductive parameters, providing valuable insights into the intricate interplay between caffeine, aspartame, and female reproductive physiology.

By providing a comprehensive assessment of the combined influence of caffeine and aspartame on female reproductive parameters in mice, this study aims to inform public health strategies and promote evidence-based dietary choices to protect reproductive well-being. Ultimately, the scope of the study encompasses advancing scientific knowledge to address critical gaps in understanding and contribute to efforts aimed at safeguarding reproductive health.

For future research, there are areas to explore with regard to the female reproductive system of mice. Firstly, further investigations could be carried out into the underlying mechanisms through which aspartame and caffeine exert their effects on the female reproductive system and its hormones, including molecular pathways and gene expression changes. Additionally, expanding the study to assess fertility parameters such as mating success, pregnancy outcomes, and offspring health would provide a more comprehensive understanding of reproductive

toxicity. Furthermore, considering different exposure scenarios, including varying doses and durations, will help establish dose-response relationships and elucidate potential thresholds for adverse effects. Exploring the long-term consequences of exposure to aspartame and caffeine beyond the acute effects studied here would be valuable for assessing reproductive health risks over the lifespan. Overall, continued research in this area is crucial for informing regulatory decisions and public health recommendations regarding the safety of aspartame and caffeine consumption, particularly in vulnerable populations such as women of reproductive age.

1.8 Conspectus of the study

The study begins with an introduction providing the background and rationale for investigating the combined effects of caffeine and aspartame on female reproductive parameters, highlighting the significance of understanding their potential synergistic impact. Following this, a comprehensive literature review examines existing research on caffeine and aspartame individually, identifying gaps in knowledge regarding their combined effects on female reproductive health. The study then outlines its methodology, detailing the experimental design, animal model selection, administration protocols for caffeine and aspartame, and methods for assessing reproductive parameters. Subsequently, the Analyses section presents the experimental outcomes, including data on changes in ovarian histology, estrous cycle lengths, body weight, and reproductive organ weight, with a thorough discussion of the findings. It also interprets the results within the context of existing literature, explores potential underlying mechanisms, and discusses the implications for female reproductive health and public health initiatives, while also acknowledging study limitations and suggesting directions for future research. Finally, the study concludes with a summary of key findings, their contribution to the field, and recommendations for further investigation or intervention, all supported by a comprehensive list of references.

CHAPTER 2: LITERATURE REVIEW

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The consumption of dietary substances, including artificial sweeteners and stimulants, has become increasingly prevalent in modern society. Among these substances, aspartame and caffeine stand out as widely used additives in various food and beverage products. Aspartame, a synthetic sweetener, is often utilized as a sugar substitute due to its intense sweetness and low-calorie content. Caffeine, a natural alkaloid found in coffee, tea, and other beverages, is renowned for its stimulant properties, providing a boost in alertness and energy levels.

The popularity of aspartame and caffeine stems from their ability to enhance the taste and palatability of foods and beverages, as well as their perceived benefits, such as weight management and increased cognitive function. However, concerns have been raised regarding the safety of these substances, particularly in relation to their effects on human health, including the reproductive and neurological systems.

In recent years, a significant body of research has emerged exploring the potential health implications of aspartame and caffeine consumption. Studies have investigated their individual effects on various physiological processes, as well as their combined impact when consumed together. Understanding the findings of these studies is essential for elucidating the potential risks associated with the widespread use of these additives and informing public health policies and dietary recommendations.

2.1 Studies on Caffeine in General: Global scenario

In humans, caffeine is known to increase anxiety, nervousness, and tension while it decreases performance on fine motor tasks (Baldwin & File, 1989). In addition, it can enhance performance in many learning and cognitive tasks.

Because of the technological limits of decaffeination, tea polyphenols, good antioxidants derived from tea (Panza *et al*., 2015), are always stained with some caffeine, limiting their applicability in the pharmaceutical and functional food fields. Caffeine has pharmacological effects depending on the dose, according to Barone and Roberts (1996).

Caffeine-containing beverages should be avoided by those with high blood pressure and coronary heart disease because they can raise blood pressure and disturb normal cardiac rhythm (Wanyika *et al*., 2010).

Caffeine promotes mental alertness and mood (Lieberman, 2001). It also reduces the risk of chronic diseases such as type 2 diabetes, liver ailments (liver damage, cirrhosis, hepatocellular carcinoma), Parkinson's disease, colon cancer, and coronary heart disease (CHD), and improves immunological processes in the human body. However, the cause of these physiological changes is unknown (Andersen *et al*., 2006; Greenberg *et al*., 2007).

Caffeine consumption exceeding 400 mg/day, according to Tucker (2003), has negative health consequences in various age groups, such as adults and persons with hypertension. Caffeine use in excess of 744 mg/day increases urine calcium excretion. Inadequate dietary calcium has been found in studies to produce more significant bone deterioration (Ilich & Kerstetter, 2000). Caffeine use increases the likelihood of developing bone disorders such as osteoporosis (Hallstrom *et al*., 2006), alveolar bone loss (Bezerra *et al*., 2008), and disruption in the early phases of bone repair (Duarte *et al.*, 2009). Women who acknowledged to consuming five or more cups of caffeinated coffee per day had a statistically significant greater risk of ovarian cancer when compared to non-consumers of coffee (Lueth *et al*., 2008).

Caffeine has been proved to have a direct stimulatory impact, increasing systolic and diastolic blood pressure by 10 - 15 mm Hg, presumably via the renin-angiotensin system (Kost *et al*., 1994). It has been observed to reduce pulse rate by 2-5 beats per minute (Battig, 1992) and to cause arrhythmias (Prineas *et al*., 1980) and tachycardia (Dobmeyer, 1983).

Caffeine-induced myocyte separation, sarcolemmal membrane damage, and contraction band necrosis were all seen in morphological studies of caffeine-injured rats (VanderHeide and Ganoto, 1985). According to Nikodijević *et al.* (1993) chronic caffeine consumption in mice reduced locomotor exploratory activity, necessitating four days of withdrawal to restore normal levels. The stimulatory effects of injected caffeine were lower in chronically treated mice, and the dose-response curve for injected caffeine was shifted. Chronic caffeine also enhanced the

depressant effects of A1- and A2-selective adenosine analogs, while reducing the stimulatory effects of dopaminergic agents.

In a study carried out by Sanday *et al.* (2013) mice were given caffeine before training and testing, causing anxiogenic effects and impaired memory retention. Pre-training administration did not alter learning but counteracted the deficit. The study highlights the importance of considering state dependency in interpreting the cognitive effects of caffeine and the possible involvement of caffeine-induced anxiety alterations in memory deficits.

According to Narishige *et al.* (2014) caffeine alters the circadian rhythm in mice by prolonging the period and causing peripheral clocks to change phase. These findings imply that consuming caffeine with meals or drinks may aid in the food-induced resetting of peripheral circadian clocks.

The study conducted by Gubareff and Sleator (1965) found that caffeine has a positive inotropic effect on guinea-pig atria, increasing transmembrane action potentials (AP) duration and contraction strength. However, the effect is not due to caffeine releasing endogenous catecholamines, but rather the presence of calcium in the external medium. Caffeine also antagonizes the depressant effects of adenosine on contraction and action potentials in guinea-pig and human atrial muscle, possibly by changing calcium's functions within the cell.

According to Schlegel and Pardee (1986) caffeine induces mitotic events in mammalian cells before DNA replication (S phase). This occurs after entering S phase and when DNA synthesis is inhibited by over 70%. Caffeine induces the translation or stabilization of mitosis-related RNA, which accumulates in S-phase cells when DNA replication is suppressed.

Si *et al.* (2005) concluded that caffeine affects honeybee behaviour, reducing learning age in young bees and improving motivation and cognitive performance in older bees, suggesting its potential application in mammalian systems.

The study by Srinivasan and Kesavan (1977) reveals caffeine's potent growth inhibitory effects on housefly larvae, affecting puparium formation and hormone-controlled responses. High caffeine concentrations lead to marked pupal mortality.

For older women, blood pressure response is augmented, and subjective feelings of behavioural mood state are attenuated to a great degree following acute caffeine ingestion (Arciero & Ormsbee, 2009).

2.2 **Studies on Caffeine in General: National scenario**

Mathavan (1989) studied the effects of caffeine and theophylline on the silkworm *Bombyx mori* and concluded that the number of eggs produced by *B. mori* reduced in all test samples. The caffeine group increased the pupal efficiency and it expended less energy on metabolism.

Poddar (2001) stated that long-term caffeine treatment may suppress the growth of EAC cells by modulating the adrenal ascorbate level as well as corticosterone status, inhibit EAC cell-induced oxidative damage caused by reactive oxygen species by its scavenging property and suppress the EAC cell-induced induction of whole brain GABAergic activity in mice. Arora (2004) concluded that caffeine ingestion is associated with a rise in systolic and diastolic blood pressure in individuals and, therefore, may affect both the diagnosis and treatment of hypertension.

In a study conducted by Das (2016), the kidney showed a loss of intercellular material in caffeine-treated rats. The study by Vanithakumari (2016) reveals that caffeine treatment in adult albino rats resulted in significant weight loss in the testis. High-dose caffeine and combination drug treatments reduced the wet weights of the vas deferens, seminal vesicles, and ventral prostate. Caffeine also caused a reduction in serum testosterone levels, but increased gonadotropin levels. Caffeine caused degeneration in testes, necrotic changes, and a decrease in spermatogenesis.

Kumari (2016) found that caffeine did not significantly alter rats' body weight, but at high doses, it decreased testis, caput, and cauda epididymal weights, reduced sperm concentration, and stimulated sperm motility. Histologically, both caput and cauda epididymal regions were adversely affected in all groups treated with caffeine.

According to Kale and Reddy (2017), caffeine has a negative impact on appetite levels. Smoking was observed to worsen the effect of caffeine on appetite.

According to Edward *et al.* (2019), the frequency of caffeine use among medical students increases during exam days while it remains moderate to low on regular days, and the most common side effect associated with such intake was found to be nervousness and loss of sleep.

2.3 Studies on Female Reproductive Toxicity of Caffeine

Caffeine, a methylxanthine found in various commonly consumed beverages such as coffee, tea, and soft drinks, has been the subject of extensive research due to its stimulant properties and widespread use (Jones & White, 2018). While the effects of caffeine on the nervous system, cardiovascular health, and metabolism have been well-documented, its specific impact on the female reproductive system is an area that demands nuanced exploration.

Studies exploring caffeine's impact on reproductive health have primarily focused on menstrual cycle irregularities, fertility, and pregnancy outcomes (Roberts *et al*., 2017). Several studies have investigated the relationship between caffeine consumption and menstrual cycle irregularities. Jones and Smith (2017) found that elevated caffeine intake was associated with disruptions in menstrual cycle regularity, potentially influencing the hormonal milieu crucial for reproductive health. Furthermore, caffeine's potential effects on fertility and pregnancy outcomes have been subjects of considerable interest and investigation. Research by Roberts *et al.* (2017) suggested that high caffeine intake may be linked to reduced fertility and increased risk of pregnancy complications. The mechanisms through which caffeine may influence reproductive health involve its interaction with adenosine receptors, leading to alterations in neurotransmitter release and hormonal regulation (Jones & Brown, 2019).

The adenosine receptor pathway is intricately connected with the regulation of hormones such as estrogen and progesterone, crucial players in the menstrual cycle and overall reproductive function. These molecular interactions may contribute to observed changes in menstrual regularity and fertility associated with caffeine consumption.

The study by Dehnavi *et al.* (2020c) found that maternal caffeine consumption during gestation significantly reduced the mean body and ovary weight, volume, and primordial follicle population in Wistar rats' offspring. The study by Qian *et al.* (2018b) found that caffeine exposure during early pregnancy can lead to embryonic development issues, abnormal implantation, and pregnancy loss. Pregnant mice treated with moderate or high caffeine dosages experienced embryo retention, defective development, and impaired implantation. The study also revealed differences in caffeine sensitivities between mice, raising concerns about caffeine consumption during early pregnancy stages. In a study by Miao *et al.* (2007b), mice were given caffeine at different times relative to HCG, and in an in vitro study, caffeine was added to the maturation culture. In vivo maturation quality remained unchanged, while in vitro quality was negatively affected.

A study on virgin female GR mice by VanderPloeg and Welsch (1991) found that hormone treatment, starting at 8-10 weeks of age, induced mammary tumours in 95-100% of the mice. After a week of hormone treatment, caffeine was administered to half of the mice. Caffeine treatment significantly reduced the number of mammary tumours per mouse and increased the latency period of tumour appearance. Study by Fenster *et al.* (1999b) concluded that caffeine consumption may reduce menstrual duration in women. This is due to caffeine's role as a vasoconstrictor, which reduces uterine blood flow, potentially reducing menstrual bleeding and shortening menstrual duration. Studies in both animals and humans also show caffeine increases uterine vascular resistance. The study by Nagasawa and Sakurai (1986b) found that caffeine ingestion had minimal effects on vaginal opening, body growth, estrous cycle patterns, mammary gland growth, and reproduction, except for the rearing rate on day 12 of lactation. According to the study, five out of 12 mothers given caffeine lost all pups before day 12 and resulting in 51% of the rearing rate, compared to 91% for the control group. Chronic heavy caffeine intake leads to high pup mortality during early lactation.

According to James and Paul (1985), caffeine consumption during pregnancy can lead to retarded fetal growth and increased postnatal mortality. The FDA issued a warning in 1986, but later studies suggest moderate doses may not cause such effects.

Reports by Tye *et al.* (1993) said that breathing abnormalities have been speculated, with a dose-dependent increase in cleft palate, high maternal deaths, resorptions, gross facial hematomas, and club feet in mice following caffeine administration. However, moderate doses may not produce such effects.

A study by Gradstein *et al.* (1993) examining caffeine use in 1,050 women with primary infertility and 3,833 women who gave birth between 1981-1983 in the US and Canada found a significant increase in the risk of infertility due to tubal disease or endometriosis for upper levels of caffeine intake. For tubal infertility, a relative risk of 1.5 was found for those who consumed more than 7 g of caffeine per month, while for endometriosis, a risk of 1.9.

The study by Pollard and Claassens (1992) investigated the effects of caffeine exposure on birth weight differences in the second (F2) generation bred from caffeine-exposed F1 females. Results showed that caffeine did not affect sexual receptivity, fertility, gestation length, parturition, nesting activity, maternal behaviour, and reproductive senescence in F1 mothers, but prolonged parturition jeopardized the viability of the F2 generation. The study concluded that a changed genetic program delayed parturition progression compromised F1 mothers, and increased offspring mortality.

A study by Kwak *et al.* (2017) on immature female rats found that high caffeine exposure significantly delayed vaginal opening and elevated serum estradiol levels. Estradiol secretion and aromatase expression increased in ovarian cells, suggesting that peripubertal exposure to high caffeine may disrupt the hypothalamo-pituitary-ovarian axis, potentially interfering with sexual maturation in these animals.

Srinivasan and Kesavan (1979) concluded that Houseflies fed with caffeine showed significant changes in fecundity and oviposition patterns, with reduced hatchability of eggs and abnormal ovaries. Histological studies revealed blocked differentiation of oocytes, and female flies

accumulated haemolymph protein, while male flies showed decreased haemolymph protein. Ovaries had less RNA and protein than controls.

Furuhashi *et al.* (1985) investigated the effects of caffeine on pregnant women. The study included 9,921 healthy pregnant women with a gestational age of less than 24 weeks. Women who consumed more than 5 cups of coffee per day were more likely to have an imminent abortion, early labour, and fetuses that were tiny for gestational age. Pregnant women who drank a lot of coffee had a higher incidence of spontaneous abortion, chromosomal abnormalities, and congenital multi-anomalies.

2.4 Studies on Aspartame in General: Global scenario

According to researchers and physicians studying the adverse effects of aspartame, the other chronic illnesses can be triggered or worsened by ingesting aspartame (Barua and Bal, 1995). Tsakiris (2006) found that low concentrations of aspartame metabolites (10 mg/kg) had no effect on membrane enzyme activity, but high or hazardous amounts (200 mg/kg) reduced membrane AChE activity partially or significantly. Furthermore, excessive or hazardous quantities of sweetener metabolites may be associated with neurological disorders such as learning and memory functions. Simintzi (2007) expressed a similar viewpoint, reporting that low quantities of aspartame (10 mg/kg) components had no impact on hippocampus and pure acetylcholine esterase (AChE) activity, however high or hazardous concentrations of aspartame (150 mg/kg) significantly lowered both enzymes and induced muscarinic receptor associated symptoms. Christian *et al*. (2004) observed that long-term aspartame (250 mg/kg/day) ingestion can influence T-maze performance in rats, which may be related to changes in receptor density in the brain. In addition to the increased density of muscarinic receptors, persistent aspartame administration may change other receptors or neurotransmitters. According to Abdel-Salam *et al.* (2012), decreased memory function is associated with increased brain oxidative stress caused by repeated aspartame ingestion.

Many consumers have reported symptoms such as headaches (Eeden *et al*., 1994), mood disturbance (Walton, 1986), memory and cognitive impairments (Orange, 1998), urticaria (Kulczycki, 1986), fibromyalgia (Smith *et al*., 2001), panic attacks and dizziness (Drake, 1986), brain tumours (Olney *et al*., 1996), gastrointestinal disturbances and menstrual problems (Bradstock *et al*., 1986). Furthermore, several consumers and researchers stated that aspartame might worsen chronic conditions such as epilepsy (Camfield *et al*., 1992), Parkinson's disease (Karstaed & Pincus, 1993), mood disorders (Walton *et al*., 1993), and Alzheimer's disease (Roberts, 1997). Aspartame is widely used in conjunction with other dietary approaches to prevent weight gain (Malinauskas *et al*., 2006). According to Vermunt (2003), replacing sucrose with sweeteners like aspartame, mostly in drinks, leads in lower calorie intake and body weight; moreover, aspartame may be related with long-term improvement in body weight management. Aspartame has been shown to aid in the long-term maintenance of body weight loss, with users losing substantially more weight and gaining significantly less weight than individuals who did not use aspartame (Beck 2002). Many authors have described the mechanisms by which aspartame could induce reduction in the body weight.

Aspartame, according to Rogers *et al.* (1990), produces satiety in humans, which leads to weight loss. According to Hall *et al.* (2003), the satiating effect of aspartame may be due to a post-absorptive action of elevated phenylalanine levels in the blood. Aspartame breaks down into phenylalanine, aspartic acid, and methanol, accounting for 10% of the breakdown product. According to Simintzi *et al.* (2007), aspartame had no influence on enzymatic function, but the metabolites were to blame. Methanol is metabolized in the body to formate, which can subsequently be converted to formaldehyde, diketopiperazine (a carcinogen), and a range of other extremely dangerous derivatives (Clarke, 2000).

Due to its ability to cross the blood-brain barrier, aspartame is a dipeptide that can enter the bloodstream and circulate throughout the body, including the brain. Humphries *et al.* (2007) has reported that aspartame disrupts a number of processes in the body, including the metabolism of amino acids, protein structure and metabolism, nucleic acid integrity, neuronal

function, endocrine balances, and changes in catecholamine concentrations in the brain. Additionally, exposure to aspartame may result in excitotoxicity in the brain, which could lead to neuronal death following an imbalance of neurotransmitters and hormones (Olney, 1988).

Aspartame intake has been linked to a variety of central nervous system symptoms, including seizures (Camfield *et al.*, 1992) and memory loss (Moser, 1994). According to Maher and Wurtman (1987), aspartame ingestion may trigger neurological or behavioural problems in some persons. Aspartame treatment caused hypothalamic neuronal necrosis in young mice given oral dosages (Reynolds *et al*., 1976).

Dow-Edwards *et al.* (1989) treated pregnant guinea pigs during gestation and found that aspartame disrupted odour-associative learning in the pups. Aspartame pre- and postnatally given to rats resulted in impaired cognitive function in the offspring (Brunner *et al.*, 1979).

Gallus *et al.* (2007) published the results of a case-control study network on the potential link between artificial sweeteners (including aspartame) and cancer. The scientists also examined individuals with proven malignancies of the oral cavity and pharynx, oesophagus, colon, rectum, larynx, breast, ovary, and prostate before concluding that artificial sweeteners do not increase the incidence of the aforementioned diseases. Furthermore, statistical examination of the findings suggests that at low concentrations, aspartame is not significantly genotoxic. Lim *et al.* (2006) investigated the relationship between aspartame-containing beverage intake and cancer incidence. Finally, they concluded that their findings do not support the concept that aspartame raises the risk of haematological or brain cancer.

However, Sofritti *et al.* (2004) proved that aspartame is a multi-potential carcinogenic agent when administered on a daily basis at a dose of 20 mg/kg body weight, despite the fact that this quantity was significantly below the permitted daily dose (40 mg/kg body weight), which is 50 mg/kg body weight in the United States. Aspartame has been known to include by-products that are more harmful than the original chemical. When the temperature of aspartame hits 86oF, the wood alcohol in aspartame is transformed into formaldehyde and ultimately to formic acid,

causing metabolic acidosis, according to Mehl-Madrona (2005). This may result in liver damage and the development of hepatic diseases (Ishak *et al*., 1991).

Despite numerous toxicological studies of aspartame, its effects on hepatic tissue have received little attention (Abhilash *et al*., 2011). Darwish *et al.* (2009) discovered a substantial increase in aspartame-treated rats' serum L-aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP). According to Abhilash (2011), long-term aspartame use induces liver damage, which is shown by a rise in AST, ALT, ALP, and -Glutamyl transpeptidase activities in blood, as well as significant histopathological alterations mostly through the glutathione dependent system. At the permitted dose level (40 mg/kg), aspartame generated oxidative stress in the liver and kidney of male albino rats, with liver tissue being more susceptible and immediately impacted than renal tissue (Iman, 2011). According to Azza *et al.* (2012), aspartame intake causes histological lesions in the liver as well as hepatic changes.

2.5 Studies on Aspartame in General: National Scenario

Nair (2013) demonstrated that long-term aspartame intake can alter biochemical variables and the antioxidant defence system. High doses increase serum bilirubin concentration and liver enzymes and decrease glutathione peroxidase, glutathione reductase, and reduced glutathione concentrations. Histopathological studies show leukocyte infiltration and brain congestion. High doses also decrease acetylcholine esterase and Na^+, K^+-ATPase activity, sodium and potassium concentrations, and dopamine synthesis. Devi (2018b) concluded that aspartame-induced oxidative stress in brain leading to the suppression of body systems. Moreover, aspartame-induced oxidative stress promoted apoptosis in brain.

According to Devi (2018c), the present study evidently concludes that aspartame exposure increased free radical production resulting in an imbalanced 128 antioxidant status in the immune organs. Aspartame induced oxidative stress in immune organs lead to suppression of non-specific and specific immune response. The study by Devi (2018a) found that aspartame exposure significantly increased blood glucose levels and glycated haemoglobin levels in

diabetic animals, with longer durations of aspartame consumption causing marked changes. The lipid profile of diabetic animals was also altered, possibly due to a deficiency in insulin. Liver enzymes and antioxidant activity also showed significant changes. The study concluded that prolonged aspartame consumption leads to an imbalanced antioxidant status in the liver and kidney, potentially causing further complications of diabetes. The study suggests aspartame consumption is not recommended for diabetic subjects. The study by Mukherjee (2003) evaluated the mutagenic and genotoxic potentials of saccharin, aspartame, and acesulfame K in plant, animal, and bacteria test systems. The results showed that the sweeteners showed varied degrees of responses towards the test systems, with chromosomal aberrations increasing with increased duration. Raghavan (2006) found that RBC, WBC, Haemoglobin and Haematocrit showed a declining trend as the doses of aspartame increased in albino rats. The sections of liver and kidney showed degenerative changes. Ashok *et al.* (2013) observed changes in the locomotor and their anxiety levels to substantiate that long-term aspartame consumption can alter the behaviour.

2.6 Studies on Female Reproductive Toxicity of Aspartame

Aspartame, an artificial sweetener commonly used as a sugar substitute in various food and beverage products, has been the subject of extensive research and ongoing debates regarding its safety, particularly in relation to reproductive health (Doe & Smith, 2021). While regulatory bodies have approved its consumption, concerns persist, warranting a closer examination of its effects on the female reproductive system.

Existing studies on aspartame have predominantly focused on its potential role in reproductive toxicity and developmental outcomes. Johnson *et al.* (2019) explored the impact of aspartame exposure on the development of reproductive organs and fertility in animal models. Their findings indicated potential adverse effects on reproductive tissues, emphasizing the need for continued scrutiny. Additionally, research has highlighted concerns about the potential impact

of aspartame on hormonal regulation, with implications for the female reproductive system (Brown *et al*., 2020).

The potential mechanisms through which aspartame may influence reproductive health involve its breakdown into aspartic acid and phenylalanine, which are amino acids that can cross the blood-brain barrier and potentially affect neurotransmitter function (Smith & White, 2018). This raises questions about the impact of aspartame on the intricate hormonal balance crucial for the proper functioning of the female reproductive system.

According to Chen *et al.* (2022b), regular intake of diet drinks is connected with oocyte dysmorphism, reduced embryo quality, and a lower pregnancy rate. Treatment with aspartame also inhibited antioxidative activity and increased oxidative stress in the ovaries and granulosa cells. This phenomenon is caused by an aspartame-induced decline in mitochondrial function (maximal respiration, spare respiratory capacity, and ATP production capacity) and triggered mitochondrial biogenesis (assessed by examining the energy depletion signalling-related factors sirtuin-1, phosphorylated adenosine monophosphate-activated protein kinase, peroxisome proliferator-activated receptor-gamma coactivator-1α, and nuclear respiratory factor 1 expression levels). Aspartame may interfere with fertility by sparing fewer follicles in the ovary and interfering with steroidogenesis in granulosa cells.

The study by Mostafa *et al.* (2020b) found that both MSG and ASP, either individually or mixed, significantly impacted the ovarian functions of rats. Aspartame alone did not alter estrogen levels or gene expression, but when combined, it decreased estrogen levels and gene expression, leading to reproductive dysfunction, possibly due to ovarian redox imbalance. Azeez (2021) found that ASP treatment resulted in lower levels of E2, GnRH, FSH and LH in male and female rats.

Rodrigues *et al.* (2022) said that, in rodents, aspartame ingestion during pregnancy was found to cause a reduction in placental and fetal weights. Shalaby *et al.* (2019) found that aspartame significantly decreased placental weight and thickness, causing damage to the placenta,

including rupture of the interhemal membrane and lysis of glycogen trophoblast cells. This suggests that aspartame consumption, especially during pregnancy, should be restricted.

The study conducted by Hosseini *et al.* (2021) concluded that high dose of aspartame can cause adverse effects on histomorphology, histomorphometry and expression of P53, Bcl-2 and Caspace-3 genes in rat ovaries.

Lennon *et al*. (1980) studied the effects of aspartame on reproduction and lactation and found that aspartame (APM) administration to female rats and hamsters did not affect postcoital fertility. However, higher levels of APM (7.5 and 14%) led to reduced food consumption, body weight loss in dams, and retarded growth rates in young. The effects of APM on lactating rats and their litters were not affected.

The study carried out by Naik *et al.* (2023) concluded that female mice treated with aspartame for 30-60 days experienced significant weight reduction, organ weight reduction, and gonadosomatic index changes, particularly in the 60-day group. The animals also showed a decrease in gonadotropins and steroids, as well as histopathological changes in uterine structure.

2.7 Studies on Caffeine and Aspartame in Combination

The study by Fitzpatrick, Carolyn A. (1994) examined the effects of caffeine and aspartame on male Wistar rats' food intake and anxiety-like behaviour. High caffeine and aspartame reduced food intake, but rats developed a tolerance to caffeine's anorectic effects. Low or high doses of aspartame blocked this tolerance. No anxiogenic effects were observed, but baseline anxiety levels were high. Future research should explore feeding suppression effects over extended periods.

In a study conducted by Fareed and Mostafa (2020), pregnant rats were divided into four groups: control, ASP, caffeine, and ASP & caffeine. All treatments were given orally from the first day of pregnancy until postnatal day 30. Kidneys were dissected and tested for oxidative stress markers. Results showed a significant increase in oxidative load in group IV, decreased

glutathione and antioxidant enzyme activities, delayed maturation of renal tissues, and pathological changes.

According to Mercola.com, the amino acid aspartic acid, which composes 40 percent of aspartame, elevates the level of aspartame in the blood. This chemical travels to the brain via the blood and performs as a neurotransmitter. It allows calcium and free radicals to enter brain cells that later die as a result of overstimulation; aspartame is referred to as an excitotoxin because it "excites" cells to death. Caffeine, which has side effects similar to aspartame, also stimulates brain activity and can compound the damage aspartame may cause. Taking caffeine in conjunction with aspartame may worsen side effects (Jones, 2011).

2.8 Critical Review of Published Studies on Aspartame and Caffeine

Aspartame and caffeine, commonly used additives in food and beverages, have been subjects of extensive research to understand their potential health effects. This critical review aims to evaluate the findings of published studies on the individual and combined effects of these substances, considering both supporting and opposing views, as well as highlighting limitations in the existing research.

Individual Effects of Aspartame: Several studies have investigated the individual effects of aspartame consumption on various physiological systems. Johnson *et al.* (2019) highlighted potential adverse effects on reproductive tissues in animal models, suggesting a need for continued scrutiny. Their findings indicated potential adverse effects on reproductive organs and fertility in animal models, emphasizing the importance of further investigation. However, the extrapolation of findings from animal models to humans and the determination of relevant exposure levels remain significant challenges in translating these results to human health implications.

Brown *et al.* (2020) raised concerns about aspartame's impact on hormonal regulation, particularly in the female reproductive system. While their findings suggest possible

implications, further research is needed to establish causality and assess the significance of these effects in humans. The study underscores the need for well-designed human studies to elucidate the potential risks associated with aspartame consumption, especially concerning reproductive health.

Individual Effects of Caffeine: Research on caffeine has yielded mixed findings regarding its physiological effects. While caffeine has been associated with increased mental alertness and performance enhancement in cognitive tasks (Lieberman, 2001), Baldwin & File (1989) reported negative effects on fine motor tasks and increased anxiety and tension. These conflicting results underscore the complexity of caffeine's pharmacological effects and the need for context-specific considerations.

Combined Effects of Aspartame and Caffeine: Studies examining the combined effects of aspartame and caffeine consumption have provided insights into potential synergistic interactions and health implications. Fitzpatrick (1994) observed reduced food intake in rats exposed to high levels of both substances, suggesting possible additive effects on feeding behaviour. However, limitations in study design, such as short-term exposure and animal models, limit the generalizability of these findings to human populations. While the study provides valuable insights into the combined effects of aspartame and caffeine, further research using human subjects and long-term follow-up is needed to confirm these findings and assess their relevance to human health. Fareed and Mostafa (2020) investigated the combined effects of aspartame and caffeine on oxidative stress markers in pregnant rats. Their results indicated increased oxidative load and renal tissue damage, suggesting potential adverse effects of combined exposure to these substances during pregnancy. However, the relevance of these findings to human health remains uncertain, and further studies are needed to elucidate the underlying mechanisms and assess potential risks. The study highlights the importance of

considering the combined effects of common food additives in assessing their impact on health outcomes, particularly during vulnerable periods such as pregnancy.

Limitations and Future Directions: Despite the valuable insights provided by existing studies, several limitations need to be addressed to advance our understanding of the health effects of aspartame and caffeine. These include reliance on animal models, limited generalizability to human populations, short-term study durations, and challenges in assessing long-term health outcomes. Future research should prioritize well-designed human studies with longer follow-up periods to elucidate the potential risks and benefits of aspartame and caffeine consumption comprehensively.

CHAPTER 3: METHODOLOGY

3. METHODOLOGY

3.1 TEST COMPOUNDS – CAFFEINE AND ASPARTAME

3.1.1 Caffeine

Caffeine, obtained in the form of anhydrous crystals, was sourced from Hi-media with a molecular weight of 194.19 g/mol (CAS No: 58-08-2). This batch comprised 100 grams of the compound and boasted a purity level within the range of 98-102%. Visually, the caffeine appeared either colourless or as white to light yellow crystals or powder, presenting a solid physical state. With a density of 1.23 g/cm³, it exhibited solubility where 10 mg dissolved in 1 mL of water. The melting range of this caffeine batch was measured between 233 and 239°C. Its CAS number was registered as 58-08-2.

Caffeine, chemically known as 1,3,7-trimethylxanthine, is a bitter, white crystalline purine and a methylxanthine alkaloid. It is structurally related to adenine and guanine bases found in DNA and RNA. Occurring naturally, caffeine is commonly found in various plants, including coffee beans, tea leaves, and cocoa beans. Its presence in these plants serves to protect them against herbivores and competition by inhibiting the germination of nearby seeds and encouraging consumption by certain animals such as honey bees (Wright *et al.*, 2013).

a. History: Caffeine's discovery dates back to the early 19th century, with German chemist Ferdinand Runge isolating relatively pure caffeine in 1819. The word "caffeine" entered common usage around 1858. Its structure was successfully elucidated by German chemist Hermann Emil Fischer in the late 19th century, earning him the Nobel Prize in 1902 (Mandal, 2009). The use of caffeine-rich beverages like coffee and tea has a rich historical background, with coffee being popularized in the Near East in the late 16th century and coffee houses being established in Europe in the 17th century.

b. Composition and Structure: The chemical formula of caffeine is $C_8H_{10}N_4O_2$, indicating its composition of eight carbon atoms, ten hydrogen atoms, four nitrogen atoms, and two oxygen atoms (Figure 3.1). Structurally, caffeine resembles the purine ring and is a trimethylxanthine, consisting of two fused pyrimidinedione and imidazole rings with three methyl groups located at positions 1, 3, and 7. It appears as a fleecy white or long silky crystalline powder, with a unique bitter flavour and weak solubility in polar hydrophilic solvents like water and alcohol.

c. Metabolism: Upon ingestion, caffeine is rapidly absorbed by the stomach and small intestine and is distributed throughout the body. Its metabolism primarily occurs in the liver, where it undergoes demethylation to form dimethylxanthines, uric acids, di- and trimethylallantoin, and uracil derivatives (Grant *et al.,* 1987). The primary route of metabolism in humans involves 3-ethyl demethylation to paraxanthine, catalyzed by cytochrome P4501A2. Paraxanthine is the dominant metabolite in humans and is excreted in urine (Arnaud, 1993). Caffeine's half-life varies depending on factors like age, species, and liver function, typically ranging from 3 to 4 hours in healthy individuals.

d. Action: Caffeine acts as a central nervous system stimulant by blocking adenosine receptors, thereby inhibiting the effects of adenosine, a neurotransmitter involved in promoting sleep and relaxation. By blocking adenosine, caffeine increases the activity of other neurotransmitters such as dopamine and norepinephrine, leading to heightened alertness and cognitive function (Fredholm, 1995). It may also increase blood adrenaline levels, promoting a state of arousal and focus. Additionally, caffeine can elevate intracellular calcium levels and inhibit cyclic nucleotide phosphodiesterases at higher concentrations.

e. Uses: Due to its stimulating effects, caffeine finds widespread use as a eugeroic (wakefulness promoter) and mild cognitive enhancer. It is commonly consumed in various forms, including beverages like coffee, tea, and soda, as well as energy drinks, supplements, medications, and

sleep-prevention compounds. Caffeine is also utilized in pharmacological formulations and has multiple applications, such as appetite suppression, diuretic effects, and enhancement of sclera collagen quality.

f. Consumption: Coffee and tea are major dietary sources of caffeine globally, with other sources including caffeinated soft drinks, chocolate, and certain medications. The consumption of caffeinated beverages is prevalent, with approximately 80% of women in the United States ingesting caffeinated beverages daily. The amount of caffeine consumed can vary widely depending on factors such as the type of beverage and individual preferences.

Caffeine's effects can vary among individuals, and regular consumption may lead to tolerance, reducing its stimulant effects over time (Mitchell *et al*., 2014). Abrupt cessation of caffeine intake can result in withdrawal symptoms such as irritability, headache, and fatigue, although these symptoms typically subside within a week. Gradual reduction of caffeine intake may help mitigate withdrawal effects.

3.1.2 Aspartame

Aspartame was obtained in a solid state from Hi-media, with a molecular weight of 294.30 g/mol (CAS No: 22839-47-0). This batch contained 5 grams of the compound and adhered to a purity standard of ≥ 98%. The appearance of the substance ranged from white to off-white hygroscopic crystals or powder. With a density of 1.347 g/cm³, it exhibited solubility where 33.3 mg dissolved in 1 mL of 0.5M hydrochloric acid. The melting range recorded for this batch fell between 242 and 248°C. Its CAS number was noted as 22839-47-0.

Aspartame, a high-intensity low-calorie artificial sweetener, has gained widespread use as a sugar substitute in various food and beverage products. Its popularity stems from its ability to provide sweetness without the added calories associated with sugar consumption. Aspartame, with its molecular formula C14H18N2O5 and molecular weight of 294.31 g/mol, is

approximately 200 times sweeter than sucrose. It is composed of two amino acids, aspartic acid, and phenylalanine, along with methanol as a breakdown product.

a. Composition and Structure: Aspartame is composed of aspartic acid and phenylalanine, which are essential amino acids utilized by the body in protein synthesis (Figure. 3.2) It is noteworthy that only the α form of aspartame exhibits sweetness, while the β form does not contribute to its sweet taste. Methanol, a breakdown product of aspartame, poses concerns due to its potential toxicity, particularly when consumed in significant amounts. The metabolism of aspartame in the gastrointestinal tract involves enzymatic hydrolysis, resulting in the release of aspartic acid, phenylalanine, and methanol, which are subsequently absorbed into the systemic circulation (Hozayen *et al*., 2014).

b. History and Regulatory Approval: Discovered by James Schlatter in 1965, aspartame was initially introduced as a sweetening agent under various trade names such as NutraSweet, Splenda, and Equal. Regulatory bodies such as the FDA and the European Food Safety Authority have established acceptable daily intake (ADI) values for aspartame to ensure its safety for human consumption. Despite concerns raised by some studies regarding potential health risks, regulatory agencies have deemed aspartame safe for consumption within specified limits (Magnuson *et al.,* 2007).

c. Metabolism and Safety Concerns: Aspartame undergoes metabolism in the gastrointestinal tract, yielding its constituent amino acids and methanol (Rycerz *et al*., 2013). While the metabolites of aspartame are similar to those derived from common dietary sources, concerns persist regarding the potential adverse effects of methanol accumulation and formaldehyde production. Studies have suggested possible links between aspartame consumption and conditions such as neurodegeneration, phenylketonuria (PKU), and methanol toxicity. However, the evidence remains inconclusive, and further research is needed to elucidate the

underlying mechanisms and assess the significance of these findings in the context of human health.

d. Current Usage and Consumption Levels: Aspartame is widely used in various food and beverage products, including soft drinks, desserts, yogurt, and pharmaceuticals. Consumption levels vary across populations, with average daily intake values below established ADI thresholds in most cases. Despite its widespread use, ongoing debates surround the safety and health implications of long-term aspartame consumption, necessitating continued research efforts to address these concerns comprehensively.

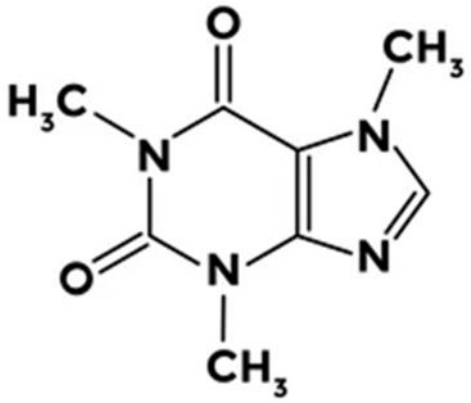

Figure 3.1 Chemical Structure of Caffeine

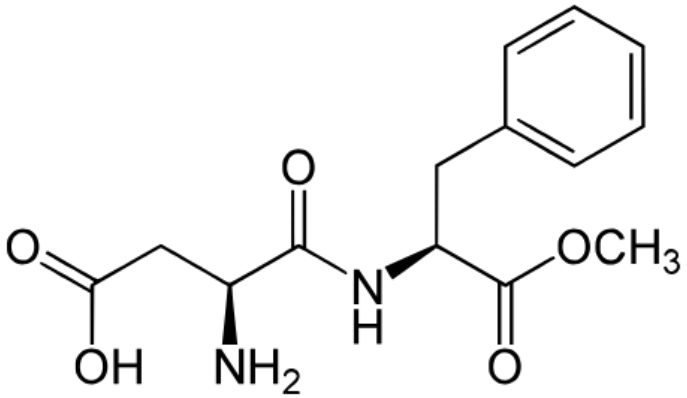

Figure 3.2 Chemical Structure of Aspartame

3.2 EVALUATION OF EFFECTS OF CAFFEINE AND ASPARTAME

3.2.1 Animal Model: Swiss Albino Mouse *(Mus musculus)*

The experimental model utilized in this study (Figure 3.3) belongs to the animal kingdom Animalia, characterized by multicellular organisms with eukaryotic cells and heterotrophic modes of nutrition. Specifically, the model belongs to the phylum Chordata, signifying the presence of a dorsal nerve cord, pharyngeal slits, a notochord, and a post-anal tail at some stage of development. Within the class Mammalia, this experimental model is classified, indicating the presence of mammary glands, hair or fur, and a neocortex region in the brain for higher cognitive functions.

Further classification places the model within the order Rodentia, which encompasses small to medium-sized mammals characterized by continuously growing incisors and a single pair of continuously growing rootless incisors in the upper and lower jaws. Within the suborder Myomorpha, this model is categorized, representing a diverse group of rodents that typically have long tails and are adapted to various ecological niches. Belonging to the family Muridae, this model is further identified, indicating membership in one of the largest families of rodents, encompassing a wide range of species distributed across diverse habitats worldwide. Specifically, the experimental model belongs to the genus Mus, which includes various species commonly known as mice. Finally, the species designation is Mus musculus, referring to the house mouse, one of the most widely used mammalian model organisms in scientific research due to its genetic similarity to humans and ease of breeding and handling.

a. Reasons for choosing the experimental model

Toxicity tests cannot be conducted on humans for ethical reasons; hence, laboratory animals are the choice for such a study. Animal studies contribute to our fundamental understanding of biology and physiology. The use of mice in this study helped me determine the effects of different concentrations or dosages of aspartame and caffeine on the female reproductive system. Swiss Albino mice, *Mus musculus* were selected for this study because they share a

significant portion of their genetic makeup with humans. They are small, easy to handle, house, and maintain, as well as adapt well to their surroundings. They are mammals and have many similarities to humans in terms of anatomy, physiology, and genetics. They are suitable for studying the toxicity in reproductive systems because they are small and highly fertile, have a short gestation period as well as an oestrus cycle, and can produce large litters. Albino mice lack pigmentation in their fur, skin, and eyes, which makes it easy to observe various phenotypic traits and changes. Besides this, female mice are widely used in research and have a rich social life. Like other rodents, their behaviour and social communication depend upon certain characteristics like sex, age, hormonal status, etc. All experiments were carried out in accordance with the guidelines and regulations established by the Institutional Animal Ethical Committee (IAEC). (IAEC Approval Ref.No.GUZ/IAEC/23-24/N5).

Figure 3.3 Animal Model: Swiss Albino Mouse *(Mus musculus)*

3.2.2 Procurement of animals

A total of 36 healthy female Swiss Albino mice *(Mus musculus),* aged 6-8 weeks with an average body weight ranging from 30-38g, were procured from The National Institute of Biosciences in Pune, India. Prior to the commencement of experimentation, the essential ethical clearance was obtained from the Animal Ethics Committee of Goa University (Reference No. GUZ/IAEC/23-24/N5, dated 12/09/2023) for the utilization of these animals.

3.2.3. Maintenance of animals

All 36 female Swiss albino mice were housed at the animal house facility of the Zoology Discipline, School of Biological Sciences and Biotechnology, Goa University. The mice were randomly separated in different polypropylene mice cages with stainless steel lid, bedded with paddy husk and was fed *ad libitum* with pellet feed and tap water. The mice were allowed to acclimatize to ambient laboratory conditions (temperature: 29-31°C; humidity: 66-69%; photoperiod: 12L:12D) for a period of 10 days (Figure 3.4). The bedding was changed on every alternate day.

3.2.4 Experimental set-up

The mice were randomly assigned to Control and Experimental groups for two exposure periods, i.e., 15 days and 30 days. Each exposure period had one Control group and two treated groups. There was a total of 6 groups, comprising 6 individuals in each group. The Experimental groups received two doses of caffeine and aspartame in combination via oral gavage administration for 2 different exposure periods. The Experimental group 1 received a lower dose of caffeine and aspartame and the Experimental group 2 received a higher dose of caffeine and aspartame for both the exposure periods (Naik *et al.,* 2023). The dose was determined based on ADI of Caffeine and Aspartame and based on a literature review. A dose of 20mg/Kg BW and 50mg/Kg BW of Caffeine and Aspartame, respectively, was administered to the mice in Experimental Group 1. A dose of 40mg/Kg BW and 100mg/Kg BW of Caffeine and Aspartame, respectively, was administered to mice in Experimental Group 2. Experimental 1 dose of Caffeine and Aspartame was prepared by dissolving 0.108g of caffeine and 0.27g of aspartame in 50 ml of Milli-Q water. Experimental 2 doses were prepared by dissolving 0.216g of caffeine and 0.54g of aspartame in 50 ml of Milli-Q water. Prior to the dosage, the mice belonging to the experimental groups were not provided with water. The dose was administered for a period of 15 days and 30 days at around the same time every day (Figure 3.4).

3.3 General examination

Over a duration of 15 days and 30 days, the mice underwent regular observations to assess their survival rate, behaviour, feeding, motor functions, and any morphological alternations in terms of fur quality, fur colour, skin texture and colour, tail spots, etc.

3.4 Body weight

Body weight is the total mass of an organism, including all tissues, organs, bones, and fluids. It is often used to assess the health and nutritional status of the female reproductive system in mice. Changes in body weight can affect fertility, reproductive performance, hormone levels, and hormonal regulation. Abnormalities in body weight and reproductive organ weight can indicate underlying disorders or diseases.

With the help of a standard laboratory weighing balance, the body weights of all female mice, control as well as experimental group animals were recorded at the initial day i.e. zero days and at the end of the different durations of the experiment i.e. 15 and 30 days. The values were expressed in grams. After euthanasia, the final body weight of mice was recorded (Figure 3.4). The initial and final body weight of mice belonging to each group was compared and the body weight gain in each group was calculated and compared amongst the other groups.

The body weight gain was calculated as:

Body weight gain = Final body weight - Initial body weight.

3.5 Estrous Cycle

Understanding the estrous cycle helps researchers monitor and assess the reproductive function of mice, providing valuable information about ovulation timing, fertility, and optimal mating periods. Abnormalities in the estrous cycle can indicate reproductive disorders or diseases. Exposure to chemicals like aspartame and caffeine and understanding the estrous cycle is crucial for assessing the efficacy and safety of these chemicals when ingested at different stages of the reproductive cycle. Toxicity studies help evaluate the potential effects of various

substances on the reproductive health of female mice, with changes in the estrous cycle serving as indicators of reproductive toxicity. Toxic substances can disrupt the endocrine system, including hormones that regulate the estrous cycle, leading to irregularities in the timing and progression of the reproductive cycle.

The estrous cycle was monitored daily (Figure 3.5) by assessing vaginal cytology to identify the distinct stages of proestrus, estrus, metestrus, and diestrus (Byers *et al.,* 2012). Vaginal lavage was collected from each mouse across all experimental groups. A smear of the lavage was prepared on a slide and examined to determine the stage of the estrous cycle. Each mouse was gently restrained using the non-dominant hand, securely grasping the scruff of the neck with the thumb and forefinger while tilting the mouse back into a supine position. The tail was held to expose the vagina, and a pre-labelled slide was prepared for each animal. A sterile dropper filled with saline was inserted no more than 0.5 cm into the vagina, and the solution was gently expelled to avoid introducing air into the vaginal cavity. The vaginal cells were collected with the saline solution and drawn back into the dropper before being expelled into the vaginal cavity again for collection. This lavage process was repeated three times to collect vaginal cells. One to two drops of the collected solution containing vaginal cells were placed onto a marked slide to make a vaginal smear, which was air-dried. Once dried, the slides were fixed by immersing them in methanol for 1 minute and allowed to air dry. Subsequently, the slides were stained with a 10% Giemsa stain solution, prepared by dissolving 10 mL of Giemsa stain stock solution in 90 mL of phosphate buffer. The excess stain was washed off with phosphate buffer. After drying, the stained slides were examined under a light microscope at both low (10x) and high (40x) magnifications to identify and record the stages of the estrous cycle based on vaginal cytology. The predominant cell types were recorded and the estrous cycle stage for each mouse was classified (Vidal & Filgo, 2017). This process was repeated daily to track the changes in vaginal cytology and determine the duration of each cycle.

Various cell types were used to identify the estrous cycle stages – i) Proestrus: Nucleated epithelial cells with a round shape. ii) Estrus: Cornified squamous epithelial cells. iii) Metestrus:

Along with cornified epithelial cells, there are small, highly pigmented neutrophils. iv) Diestrus: Abundance of neutrophils along with some nucleated epithelial cells.

3.6 Collection of tissues

After 15 days and 30 days of exposure periods, the mice were sacrificed by cervical dislocation, wherein pressure was exerted on the neck to dislocate the spinal column from the skull (Figure 3.4) Following this, dissection was carried out and the general viscera was exposed (Figure 3.6). The ovaries and uterus were excised and cleared off from fat tissues attached and then rinsed with cold 0.9% NaCl solution to eliminate any blood residues (Baratta *et al*., 2009).

3.7 Organ Weight

Organ weight analysis provides valuable data for assessing the physiological impact and potential risks associated with the administration of test compounds, aiding in the interpretation of their safety and efficacy profiles (Naik *et al*., 2023).

3.7.1 Ovary Weight

For both the 15-day and 30-day exposure periods, the ovaries were carefully excised, and excess fat tissue and blood were removed to ensure accurate measurement of ovarian weight (Fig 3.6). The excised ovaries were weighed using a precise balance, and the weights were recorded for each specimen. The average ovarian weights were compared across the Control group, Experimental group 1, and Experimental group 2 for both exposure periods.

3.7.2 Uterus Weight

For both the 15-day and 30-day exposure periods, the uterus was carefully excised, and excess fat tissue and blood were removed to ensure accurate measurement of uterus weight (Fig 3.6). The excised uteri were weighed using a precise balance, and the weights were recorded for each

specimen. The average uterus weights were compared across the Control group, Experimental group 1, and Experimental group 2 for both exposure periods.

3.8 GSI

Gonadal somatic index is a reproductive biology measure that evaluates the relationship between an organism's reproductive organs and its total body mass. It quantifies the investment in reproductive organs relative to the overall body mass. Changes in GSI can indicate reproductive status shifts, such as increased investment during breeding seasons, and are often linked to variations in reproductive hormones (Naik *et al*., 2023).

GSI was calculated by the following formula:

GSI = Gonad (ovary) weight (g) ÷ Body weight (g) x 100

3.9 Histological Analysis

Histopathology is a branch of pathology that involves microscopic examination of tissues to study diseases. It is particularly useful in studying ovarian tissue, which is essential for diagnosing various ovarian conditions such as tumours, cysts, inflammatory disorders, and developmental anomalies. Histopathological examination of the ovaries is crucial for studying the female reproductive system of mice, providing detailed insights into structural and cellular changes.

Ovaries were dissected out immediately and carefully after the animals were sacrificed and then washed in cold 0.9% NaCl, cleared of any attached tissues and fats, and were blotted dry using filter paper (Baratta *et al*., 2009). Dried ovaries were then weighed (in grams) quickly with the help of a weighing machine. The ovaries were then stored in labelled vials containing 10% neutral formalin. All the vials were labelled with name of the organ, date of fixation and group details (Naik *et al*., 2023). After dehydration with 70% alcohol, the samples were embedded in paraffin wax and six-micron thick sections were prepared and stained with haematoxylin and eosin by Ashwini Pathology Lab. The prepared slides were further analysed at 4x, 10x and 40x

under a light microscope. The number of follicles in the ovarian sections, belonging to different groups were also counted and recorded.

3. 10 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 9 software. The results were expressed as Mean ± Standard deviation. Normal distribution of data was checked using Shapiro-Wilk test. Significant differences amongst groups were analyzed using one-way ANOVA, followed by post-hoc Tukey's Honest Significant Difference test. Student paired t-test was carried out to analyze significant differences in the final body weight. $P < 0.05$ was considered to be significant, $P \leq 0.01$ as highly significant and $P < 0.001$ as very highly significant. The level of significance was denoted by asterisk (*).

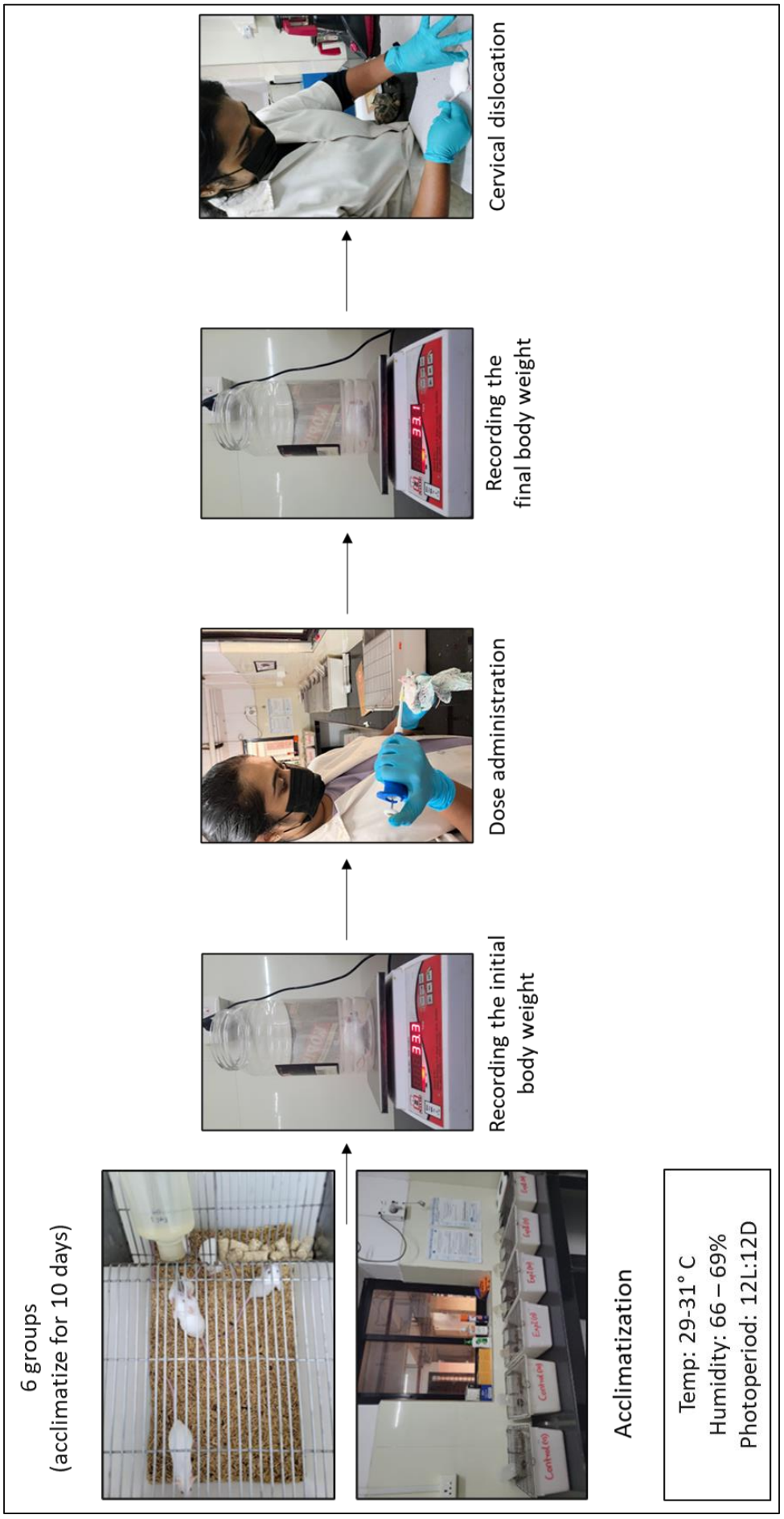

Figure 3.4 Experimental Steps (Before Euthanasia of Mice)

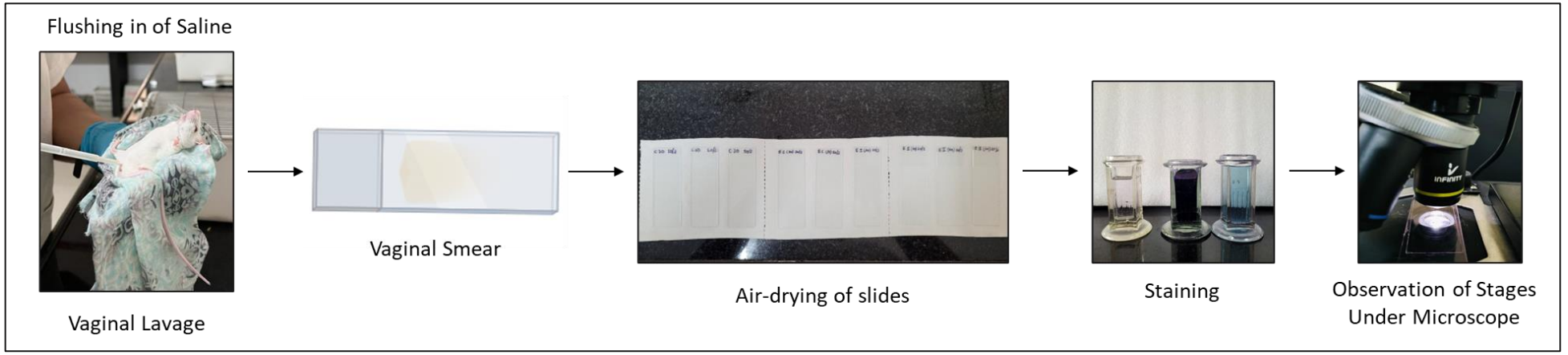

Figure 3.5 Daily Monitoring of Estrous Cycle

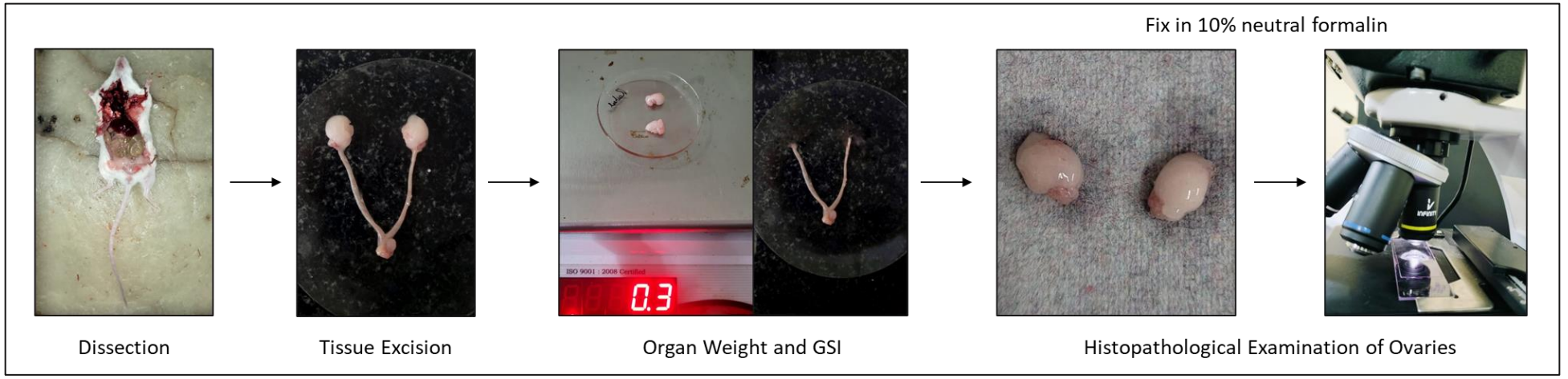

Figure 3.6 Experimental Steps (After Euthanasia of Mice)

CHAPTER 4:
ANALYSIS AND CONCLUSIONS

CHAPTER 4: ANALYSIS AND CONCLUSIONS

4.1 Results

4.1.1 Effects of Caffeine and Aspartame on the General Morphology and Behaviour of *Mus musculus*

After exposing two separate experimental groups of mice to varying doses of caffeine and aspartame over periods of 15 and 30 days, respectively, continuous monitoring was conducted to assess both morphological and behavioural changes.

Throughout the exposure periods, fur morphology, including texture, colour, and density, remained normal across all groups, with no observed abnormalities in tail spots. Behavioural analysis focused on activity levels, drowsiness, and feeding frequency. In the control group, no aberrant behavioural changes were noted. However, mice in Experimental Group 1 displayed heightened activity levels and aggressive behaviour, alongside a decrease in feeding frequency, which intensified over the exposure duration. Similarly, Experimental Group 2 mice exhibited increased activity and aggression, coupled with a reduction in feeding frequency. These mice appeared restless and demonstrated heightened alertness and vigilance.

4.1.2 Effects of Caffeine and Aspartame on Body Weight of *Mus musculus*

The body weight of mice belonging to all the groups from the 15-day exposure period and 30-day exposure period viz., Control (15), Exp 1 (15), Exp 2 (15), Control (30), Exp 1 (30), and Exp 2 (30) were recorded before the start of exposure and after the exposure periods. The weight was recorded as average initial weight and average final weight for all the groups.

The average initial weight of the mice in all 6 groups was in the range of 30-38g (Table 4.1 and Table 4.2). During the 15-day exposure period, notable differences in body weight changes were observed among the experimental groups compared to the control. The Control group exhibited a mean weight gain of 5.35 grams over the duration of the study. In contrast, the

Experimental group 1 showed a mean weight loss of 2.25 grams, while the Experimental group 2 exhibited a mean weight loss of 1.78 grams. Paired t-test was conducted to compare the initial and final body weights of mice and found that exposure to Aspartame and Caffeine over a 15-day period led to a significant increase in the final body weight among mice in the Control group ($P = 0.0005$, $t = 6.866$). The final body weight of mice belonging to the Experimental 1 group showed a significant decrease in body weight compared to its initial body weight ($P = 0.0473$, $t = 2.059$). The Experimental 2 group exhibited a further significant decrease in final body weight compared to its initial body weight ($P = 0.0004$, $t= 7.071$) (Fig 4.1).

Over the 30-day exposure period, distinct variations in body weight changes were observed across the control and experimental groups. The Control group showed a slight mean weight gain of 0.2 grams during the study duration. Conversely, the Experimental group 1 exhibited a mean weight loss of 1.46 grams, and the Experimental group 2 showed a more pronounced mean weight loss of 2.53 grams. Exposure to aspartame and caffeine over a 30-day period did not lead to a significant change in body weight among mice in the Control group ($P = 0.2694$, $t = 0.6594$) but led to a significant decrease in the final body weight among mice in Experimental Group 1 ($P = 0.0211$, $t = 2.712$). Experimental Group 2 exposure to aspartame and caffeine, resulted in a statistically significant decrease in body weight over the 30 days ($P = 0.0119$, $t = 3.204$) (Fig 4.2).

Table 4.1 Average body weight of mice in the control and treated groups before and after 15 days oral exposure to Caffeine and Aspartame.

GROUPS	BODY WEIGHT	
	Initial	**Final**
Control	30 ± 3.16	35.35 ± 1.33
Exp. Group 1	33.9 ± 4.39	31.65 ± 1.91
Exp. Group 2	37.11 ± 1.79	35.33 ± 1.63

All values represented are Mean ± Standard deviation

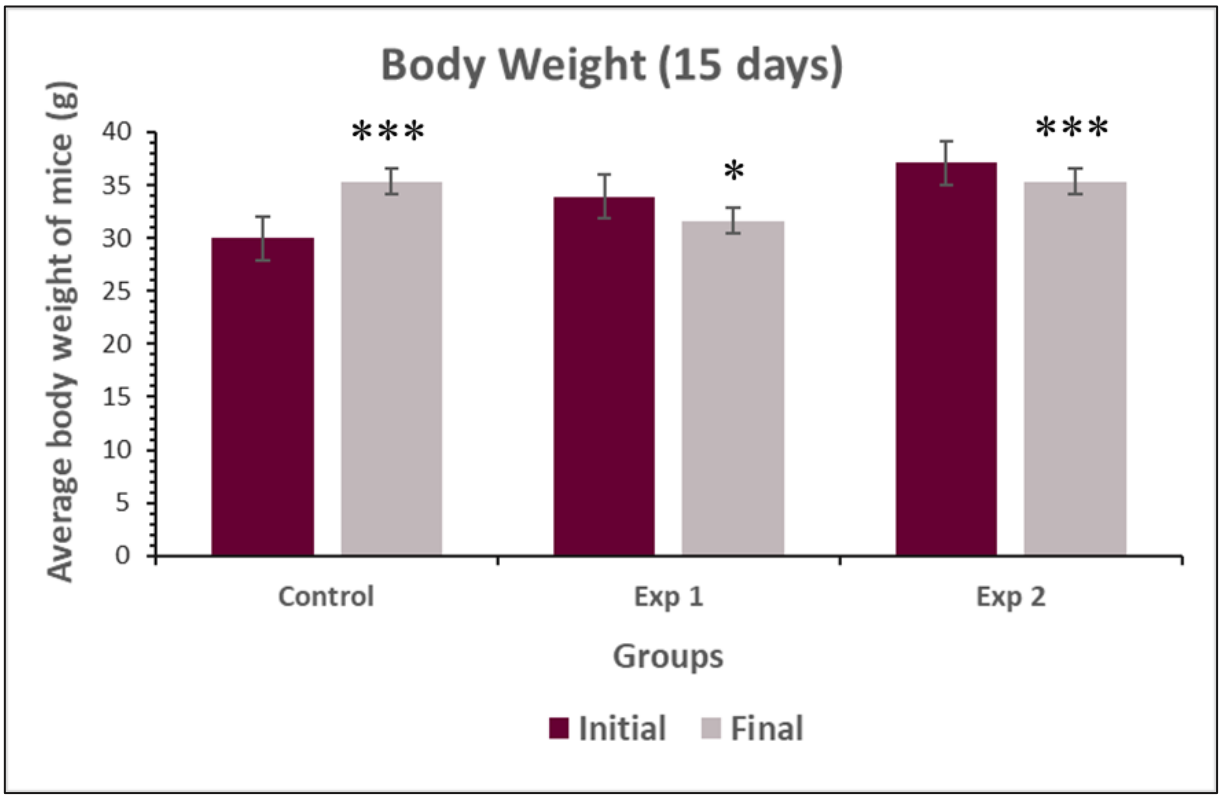

Figure 4.1 Average body weight of mice in the control and treated groups before and after 15 days of oral exposure to Caffeine and Aspartame.

Table 4.2 Average body weight of mice in the control and treated groups before and after 30 days oral exposure to Caffeine and Aspartame.

GROUPS	BODY WEIGHT	
	Initial	Final
Control	36.26 ± 3.14	36.46 ± 2.98
Exp. Group 1	32.76 ± 5.41	31.3 ± 5.81
Exp. Group 2	37.66 ± 2.83	35.1 ± 2.78

All values represented are Mean ± Standard deviation

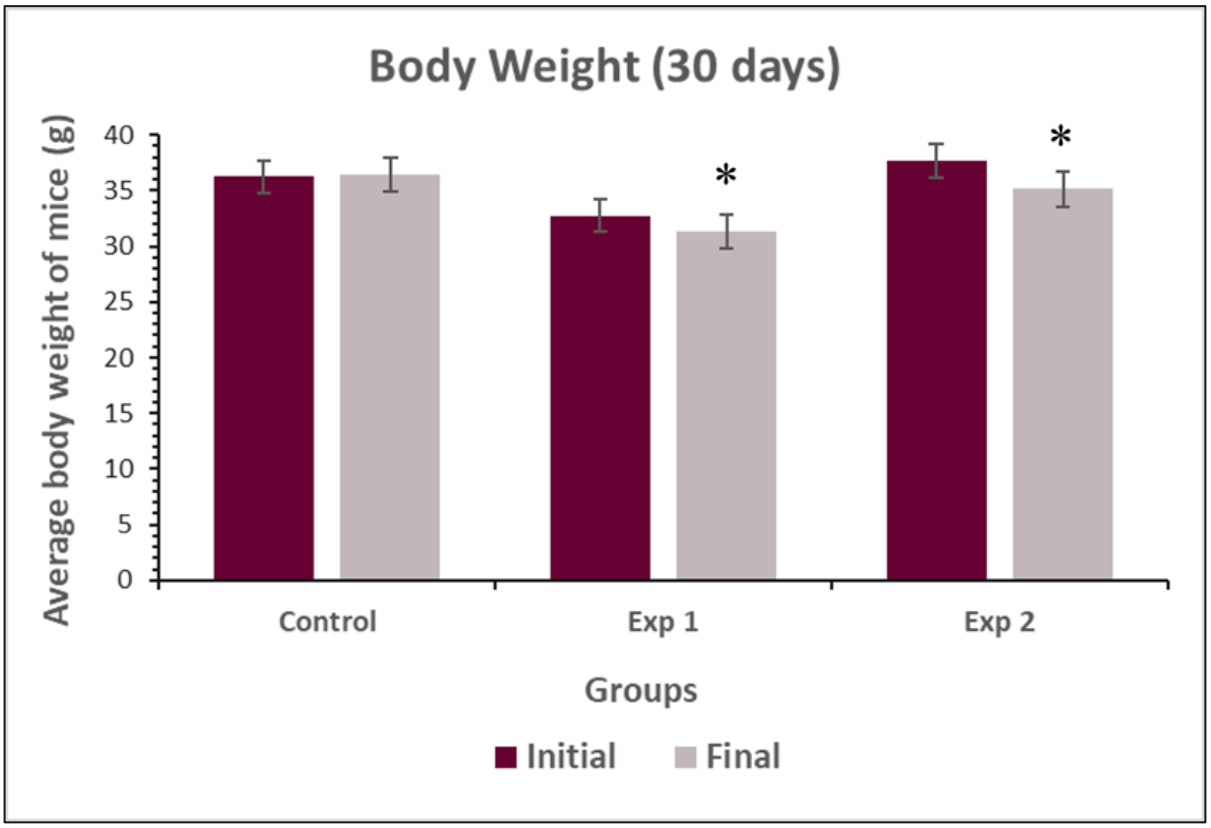

Figure. 4.2 Average body weight of mice in the control and treated groups before and after 30 days of oral exposure to Caffeine and Aspartame.

4.1.3 Effects of Caffeine and Aspartame on Organ Weight (Ovaries and Uterus) of *Mus musculus*

a. Ovary Weight

After 15 days and 30 days of exposure the mice were sacrificed by cervical dislocation and dissected for the collection of ovaries. The weight of ovaries of mice belonging to all the groups viz., Control (15), Exp 1 (15), Exp 2 (15), Control (30), Exp 1 (30), Exp 2 (30) were recorded.

There was a decrease in the average ovary weight of Experimental group 1, followed by a further decrease in the Experimental group 2 when compared to the Control group in the 15-day exposure period. The one-way ANOVA test was conducted to compare the ovary weights among the Control, Experimental 1, and Experimental 2 groups of mice and found that there was no significant difference in ovary weights among the three groups ($F = 0.60$, $P = 0.5615$) suggesting that the exposure period and the doses did not have a significant effect on ovary weight (Fig 4.3).

After a 30-day exposure period to Caffeine and Aspartame, there was a significant difference in ovary weights among the three groups ($F = 7.045$, $P = 0.0070$) where the Experimental group 2 had the least weight, followed by Experimental group 1 when compared to the Control group. Tukey's post-hoc test revealed significant differences amongst Control and Experimental group 1 ($P = 0.0269$) and highly significant differences amongst Control and Experimental group 2 ($P = 0.0085$) (Fig 4.3).

Table 4.3 Average ovary weight of mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

GROUPS	WEIGHT OF OVARIES	
	15 DAYS	30 DAYS
Control	0.28 ± 0.05	0.28 ± 0.04
Exp. Group 1	0.25 ± 0.05	0.2 ± 0.06
Exp. Group 2	0.23 ± 0.05	0.18 ± 0.04

All values represented are Mean ± Standard deviation

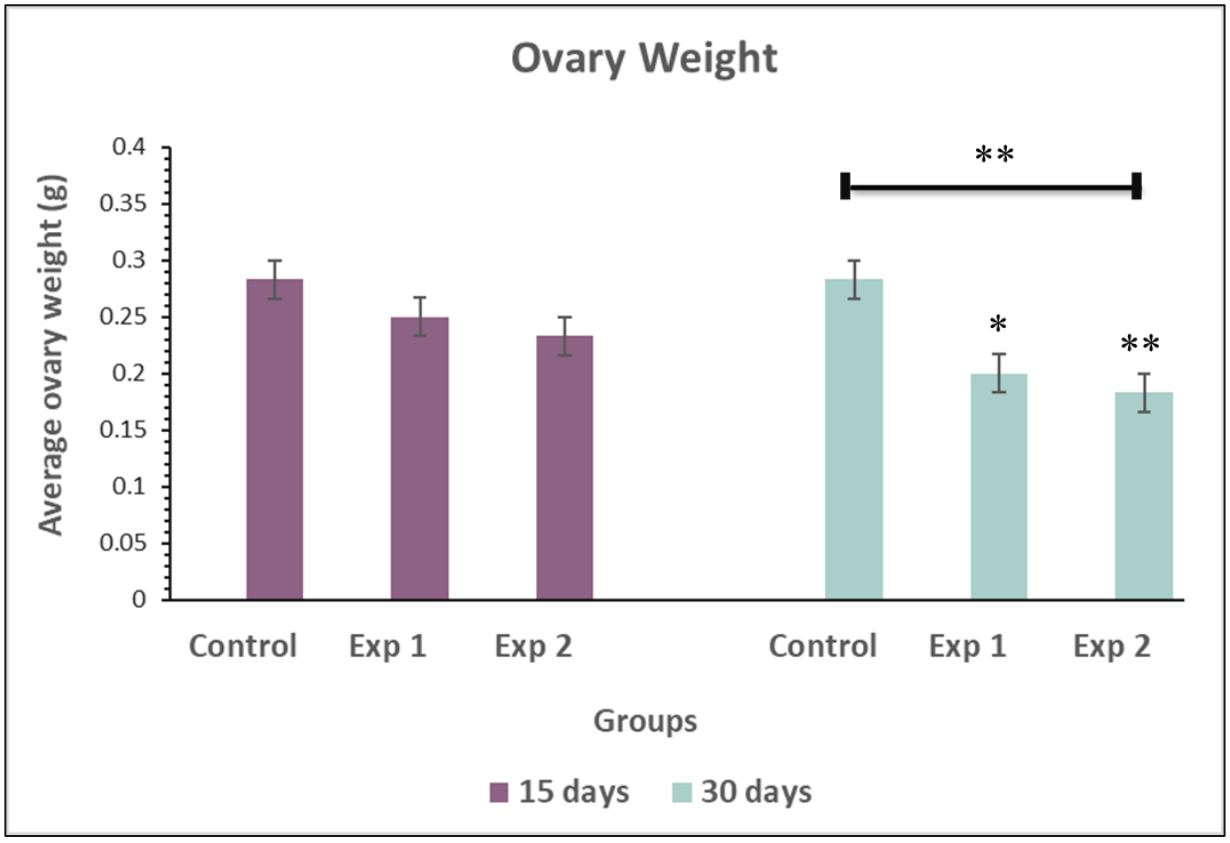

Figure. 4.3 Average ovary weight of mice in the control and treated group after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

b. Uterus Weight

After 15 days and 30 days of exposure the mice were sacrificed by cervical dislocation and dissected for the collection of uteri. The weight of uterus of mice belonging to all the groups viz., Control (15), Exp 1 (15), Exp 2 (15), Control (30), Exp 1 (30), Exp 2 (30) were recorded.

There was a decrease in the average uterus weight of Experimental group 1, followed by a further decrease in the Experimental group 2 when compared to the Control group in the 15-day exposure period. The one-way ANOVA test was conducted to compare the uterus weight among the Control, Experimental 1, and Experimental 2 groups of mice. After a 15-day exposure period to Caffeine and Aspartame, there was no significant difference in uterus weight among the three groups ($F = 1.912$, $P = 0.1821$), suggesting that the exposure period did not have a significant effect on uterus weight (Fig 4.4).

After a 30-day exposure period to Caffeine and Aspartame, there was a significant difference in uterus weights among the Control, Experimental group 1, and Experimental 2 group of mice ($F = 7.167$, $P = 0.0065$) where the Experimental group 2 had the least weight, followed by Experimental group 1 when compared to the Control group. Tukey's post-hoc test revealed significant differences amongst Control and Experimental group 1 ($P = 0.0230$) and highly significant differences amongst Control and Experimental group 2 ($P = 0.0085$) (Fig 4.4).

These findings in the differences in organ weights suggest that the duration of exposure and difference in doses had a notable impact on ovary weight and uterus weight in the experimental groups.

Table 4.4 Average uterus weight of mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

GROUPS	WEIGHT OF UTERUS	
	15 DAYS	30 DAYS
Control	0.25 ± 0.05	0.28 ± 0.04
Exp. Group 1	0.23 ± 0.05	0.18 ± 0.04
Exp. Group 2	0.18 ± 0.07	0.16 ± 0.08

All values represented are Mean ± Standard deviation

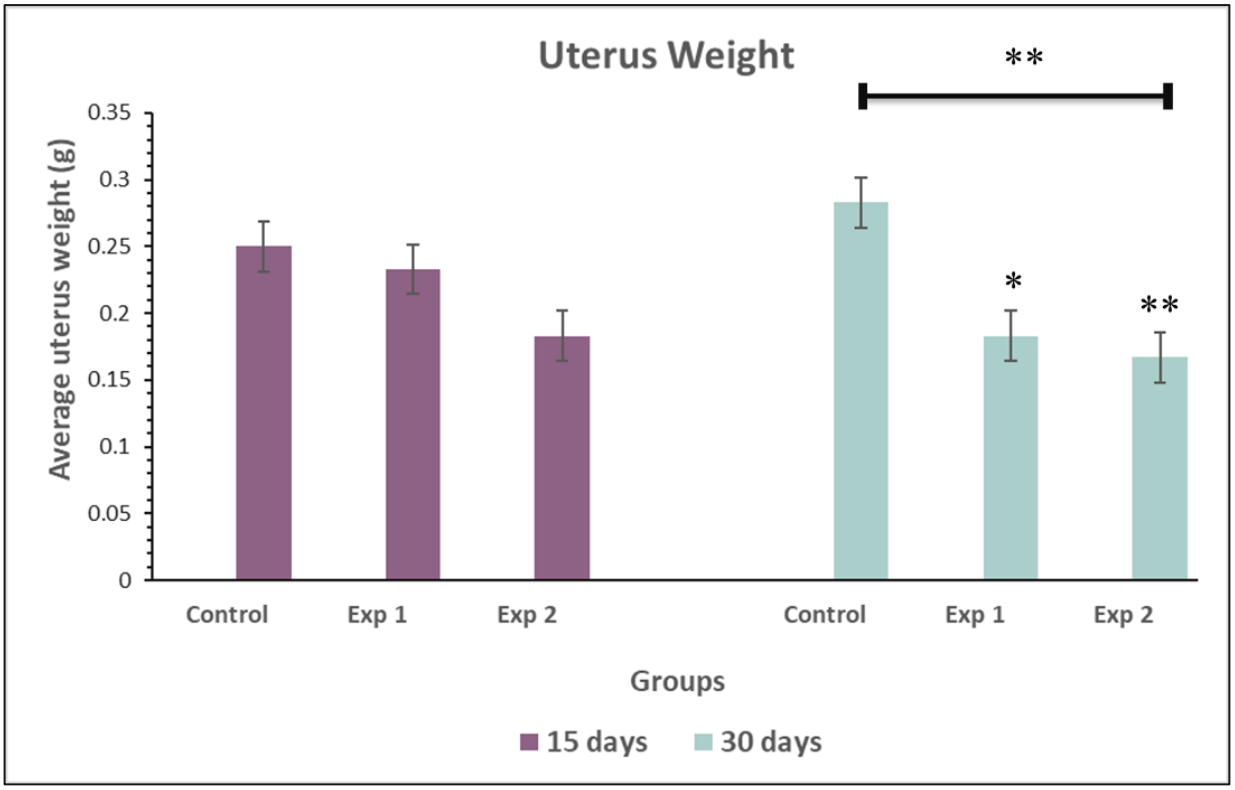

Figure 4.4 Average uterus weight of mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

4.1.4 Effects of Caffeine and Aspartame on the GSI of *Mus musculus*

After 15 days and 30 days of exposure, the body weight of mice belonging to all the groups was recorded and then sacrificed by cervical dislocation and dissected for the collection of ovaries. The ovaries of mice belonging to all the groups viz., Control (15), Exp 1 (15), Exp 2 (15), Control (30), Exp 1 (30), Exp 2 (30) were weighed and the values were recorded. With the help of the average body weight and average ovary weight values, the average GSI was obtained for mice belonging to all the groups.

There was a decline in the average GSI of Experimental Group 1, followed by a further decrease in Experimental Group 2 when compared to the Control group in the 15-day exposure period. After one-way ANOVA analysis, it was found that there is no significant difference in the GSI among the Control, Experimental 1, and Experimental 2 groups ($F = 1.924$, $P = 0.1804$) (Fig 4.5).

After the 30-day exposure period to Caffeine and Aspartame, there were significant differences in GSI among the Control, Experimental 1, and Experimental 2 ($F = 9.230$, $P = 0.0024$) where the Control group had the highest GSI, followed by Experimental group 1 and Experimental group 2 with the lowest value. Tukey's post-hoc test revealed significant differences amongst Control and Experimental group 2 ($P = 0.0018$) (Fig 4.5).

Table 4.5 Average Gonadal Somatic Indices (GSI) of mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

GROUPS	GSI (%)	
	15 DAYS	30 DAYS
Control	0.80 ± 0.0011	0.78 ± 0.0008
Exp. Group 1	0.79 ± 0.0017	0.63 ± 0.0010
Exp. Group 2	0.66 ± 0.0012	0.52 ± 0.0011

All values represented are Mean ± Standard deviation

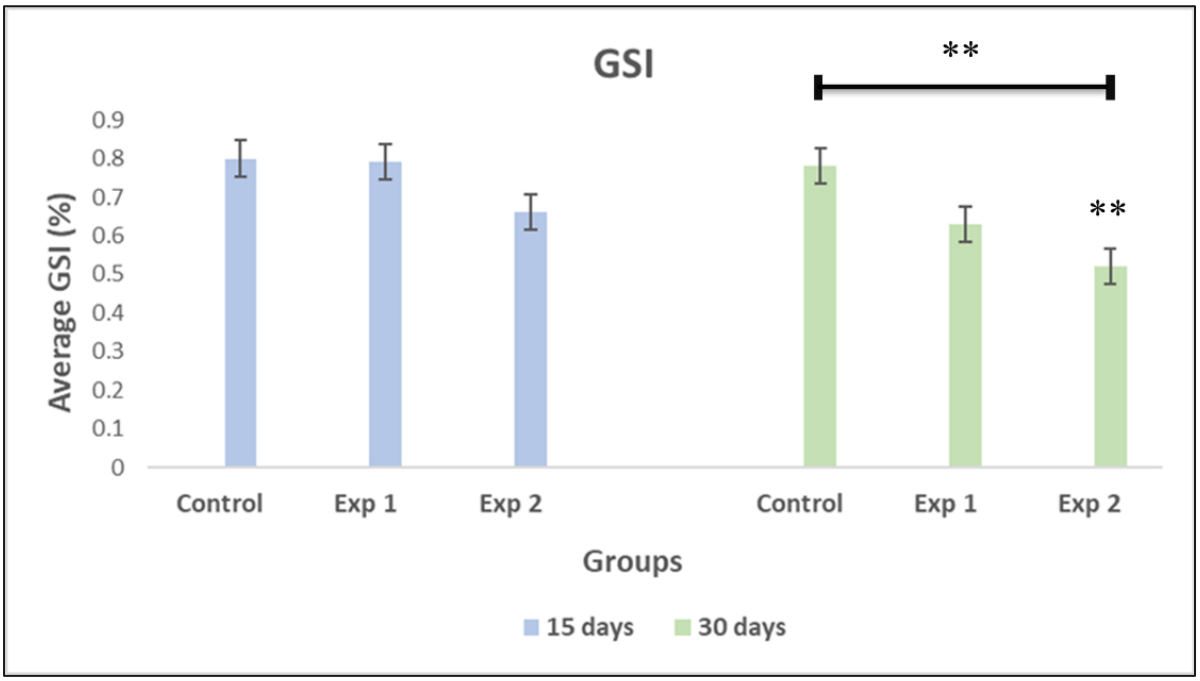

Figure 4.5 Average Gonadal Somatic Indices (GSI) of mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

4.1.5 Effects of Caffeine and Aspartame on the length of the estrous cycle of *Mus musculus*

During the 15-day exposure period, mice in the Control group, which did not receive any treatment, completed an average of 3 estrous cycles, where all four stages viz., Proestrus (Fig 4.6.A), Estrous (Fig 4.6.B), Metestrus (Fig 4.6.C), and Diestrus (Fig 4.6.D) were observed. Similar to the Control group, the mice in Experimental Group 1 exposed to a low dose of Caffeine and Aspartame, also completed an average of 3 estrous cycles within the same duration. Mice in the Experimental Group 2, subjected to a higher dose of Aspartame and Caffeine, completed slightly fewer cycles, with an average of 2.75 estrous cycles during the 15-day exposure period. Here, the last stage observed was the metestrus stage. While the control and Experimental Group 1 exhibited similar cycle completion rates, Experimental Group 2 showed a slightly lower average number of cycles completed (Fig 4.7). In the control group, the most prevalent stages observed were metestrus and estrus. Conversely, in Experimental groups 1 and 2, the estrous stage was consistently the most frequently observed phase. Initially, the estrous cycles exhibited synchrony among the Control, Experimental 1, and Experimental 2 groups. However, this synchrony dissipated over time, leading to notable changes in cycle duration and the frequency of estrous stages observed across the experimental groups.

Over the 30-day exposure period, mice in the control group completed an average of 5 estrous cycles where all four stages of the estrous cycle were observed. Mice belonging to Experimental Group 1 exposed to a lower dose of Aspartame and Caffeine completed an average of 4.75 estrous cycles. The last stage observed in the 4th cycle was metestrus. The mice exposed to a higher dose of Aspartame and Caffeine completed an average of 4.5 estrous cycles over the same 30-day period. The last stage observed was the estrous stage. Experimental Group 2 showed a slightly lower average number of cycles completed, followed by Experimental group 1 when compared to the Control group (Fig 4.7). The most frequently observed estrous cycle stage in the Control group was Diestrus. Similarly, in Experimental Group 1, Diestrus was the predominant stage. In Experimental Group 2, the most frequently observed stages were Estrus

and Diestrus. Initially, the estrous cycles in the Control, Experimental Group 1, and Experimental Group 2 were synchronized, showing similar patterns of cycle lengths and stage frequencies. However, as the study progressed, these cycles became desynchronized, exhibiting changes in both cycle lengths and the frequencies of estrous stages across the groups.

This suggests that there might be a slight effect on the estrous cycle completion in mice exposed to the experimental conditions, particularly in Experimental Group 2.

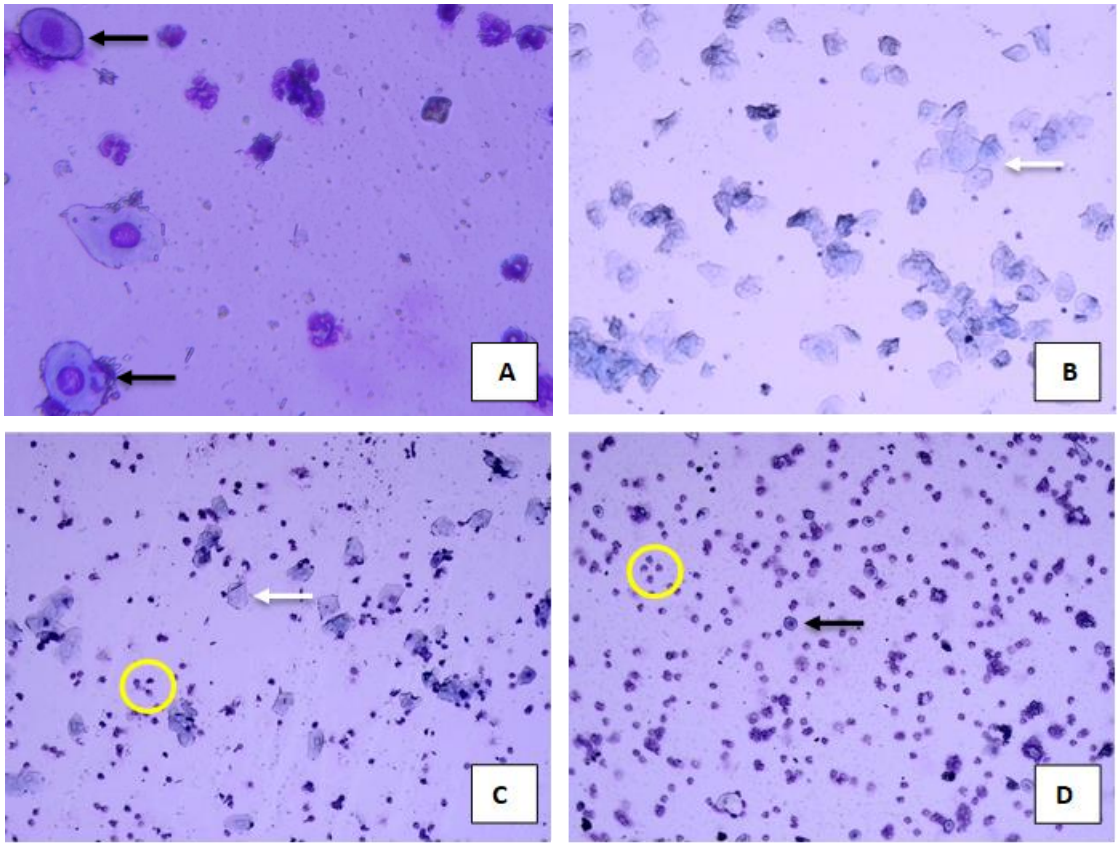

Figure 4.6 Vaginal cytology representing each stage of estrous cycle in *Mus musculus.* Three cell types are identified: neutrophils (circle), cornified epithelial cells (white arrow), nucleated epithelial cells (black arrow). Stages of estrous cycle include Proestrus (A), Estrus (B), Metestrus (C), Diestrus (D).

Table 4.6 Number of estrous cycles completed in mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

GROUPS	Number of Cycles	
	15 DAYS	30 DAYS
Control	3	5
Exp. Group 1	3	4.75
Exp. Group 2	2.75	4.5

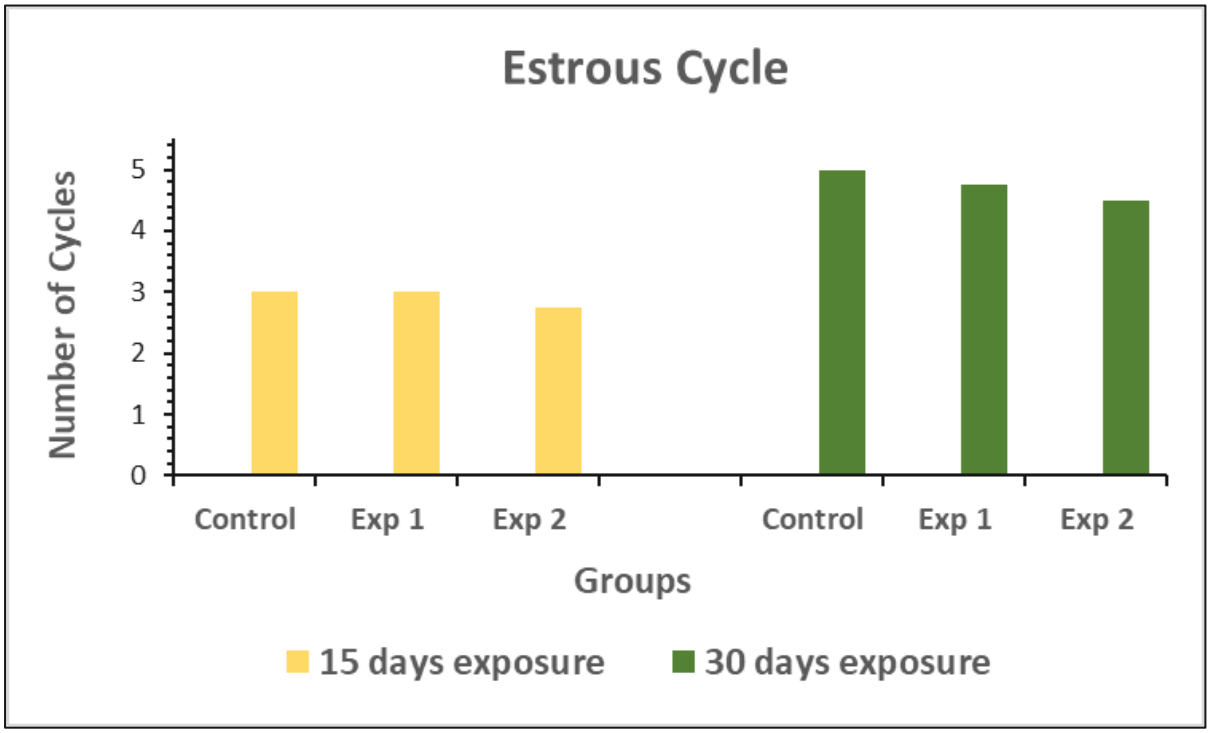

Figure 4.7 Number of estrous cycles completed in mice in the control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

4.1.6 Histological changes in the ovary of *Mus musculus*

a. Effects of Caffeine and Aspartame on the number of follicles in the ovarian section

Mice belonging to Experimental group 1 and Experimental group 2, treated with Caffeine and Aspartame showed a decrease in the follicle count compared to the Control Groups in both 15-day and 30-day exposure periods. In the 15-day exposure period, there is a notable difference in the average number of follicles in the experimental groups, with a decrease in follicles, particularly in the Experimental 2 group compared to the Control group and Experimental 1 group. This reduction in the number of follicles, especially in the Experiment 2 group, suggests a potential negative effect of Aspartame and Caffeine exposure on ovarian folliculogenesis.

Similar to the 15-day exposure period, the data recorded for the 30-day exposure period also suggests a notable difference in the average number of follicles across all stages between the Control group and the experimental groups. The Experimental 2 group had the least number of follicles when compared to Experimental group 1 and Control group.

For 30 days, the reduction in follicle numbers, particularly in the Experimental 2 group indicates a potential negative impact of Caffeine and Aspartame exposure on ovarian folliculogenesis, which appears to persist over a longer duration of exposure.

In the 15-day exposure period, there was a significant difference in the average number of primordial follicles among the three groups ($F = 7.313$, $P = 0.0061$). Both, Experimental 1 ($P = 0.0232$) and Experimental 2 ($P = 0.0076$) groups exhibited a significant decrease in the average number of primordial follicles compared to the Control group. Experimental 2 group had the least number of primordial follicles. There was no significant difference in the average number of primary follicles among the three groups ($F = 0.9459$, $P = 0.4103$). However, Experimental group 2 had the least number of primary follicles followed by Experimental group 1. With respect to the number of late primary follicles, there was no statistically significant difference in the mean number of late primary follicles among the three groups ($F = 0.5957$, $P = 0.5637$). There was no significant difference in the mean number of secondary follicles among

the three groups (F = 0.3488, P = 0.7111). There was a statistically significant difference in the average number of graafian follicles amongst the three groups (F = 5.167, P = 0.0196).

In the 30-day exposure period, a significant difference in the average number of primordial follicles was seen among the three groups (F = 3.853, P = 0.0446). Experimental group 2 exhibited a significant difference in the number of primordial follicles when compared to the control group (P = 0.0476). Experimental group 2 had the least number of primordial follicles, followed by Experimental group 1. There was no significant difference in the average number of primary follicles among the three groups (F = 2.754, P = 0.0958). A statistically significant difference in the average number of late primary follicles were seen among the three groups (F = 4.089, P= 0.0382). There was no statistically significant difference in the mean number of secondary follicles among the three groups (F = 0.2317, P = 0.7960). There was a significant difference in the number of graafian follicles between groups (F = 5.658, P = 0.0301). Experimental group 1 (P = 0.0439) and Experimental group 2 (P = 0.0185) exhibited a significant decrease in the number of graafian follicles when compared to the Control group.

These findings highlight the impact of the exposure of Caffeine and Aspartame on ovarian follicle development, with more pronounced effects observed over 30 days compared to 15 days.

In the Control group, the highest numbers of follicles were observed across all types compared to the Experimental group 1 and Experimental 2 groups. Experimental 2 group showed a significant decrease in the number of follicles across all types compared to both Control and Experimental 1 groups. The observed reduction in follicle counts, particularly in Experimental 2, suggests a potential impact of the exposure on ovarian follicle development during the 15 days. Similar trends were observed in the 30-day exposure period, with a reduction in follicle counts in the Experimental 2 group compared to Control and Experimental 1. Experimental 2 exhibited the lowest counts across most follicle types, suggesting a potential adverse effect of the exposure on follicle development over a longer duration.

Table 4.7 Average follicle count in the ovarian section of mice in the control and treated groups after 15 days of oral exposure to Caffeine and Aspartame.

Types of Follicles	Groups		
	Control	**Exp 1**	**Exp 2**
Primordial follicle	5.5 ± 1.87	2.83 ± 0.98	2.33 ± 1.63
Primary follicle	4 ± 2.19	3 ± 1.26	2.5 ± 2.16
Late primary follicle	3.16 ±1.60	2.5 ± 0.83	2.16 ± 2.13
Secondary follicle	3 ± 0.89	2.66 ± 1.96	2.33 ± 1.03
Graafian follicle	1.16 ± 0.75	0.33 ± 0.51	0.166 ± 0.40

All values represented are Mean ± Standard deviation

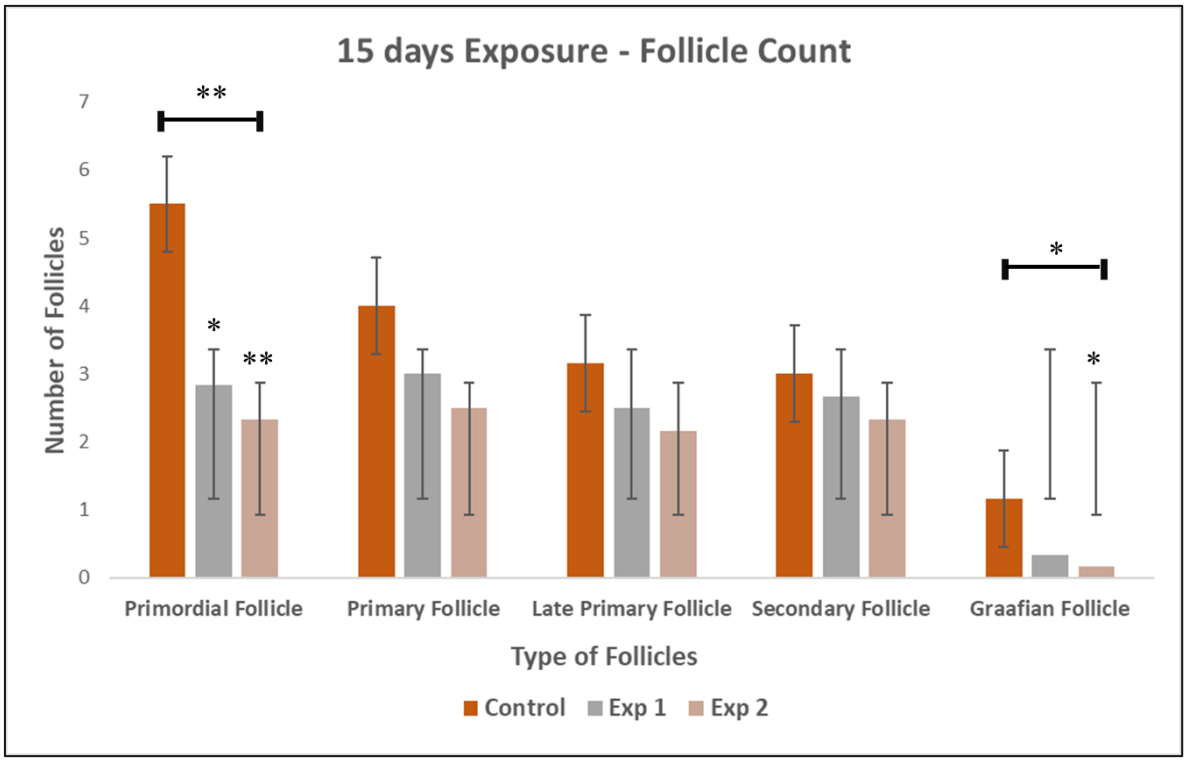

Figure 4.8 Average follicle count in the ovarian section of mice in the control and treated groups after 15 days of oral exposure to Caffeine and Aspartame.

Table 4.8 Average follicle count in the ovarian section of mice in the control and treated groups after 30 days of oral exposure to Caffeine and Aspartame.

Types of Follicles	Groups		
	Control	**Exp 1**	**Exp 2**
Primordial follicle	5.3 ± 3.01	2.66 ± 1.50	2 ± 1.78
Primary follicle	7.33 ± 2.87	4.66 ± 3.50	3.5 ± 2.16
Late primary follicle	5.16 ±1.72	2.83 ± 1.83	2.66 ± 1.50
Secondary follicle	3.5 ± 1.87	3.16 ± 1.47	2.83 ± 1.72
Graafian follicle	1.5 ± 0.83	0.5 ± 0.54	0.33 ± 0.51

All values represented are Mean ± Standard deviation

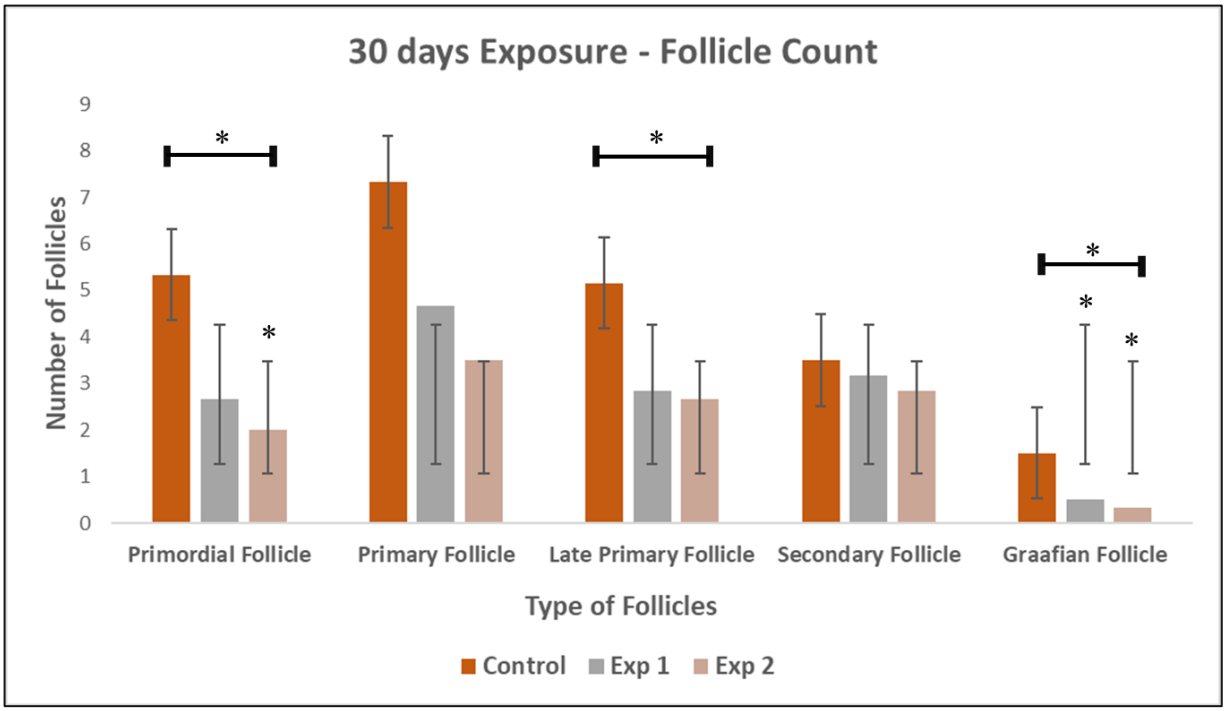

Figure 4.9 Average follicle count in the ovarian section of mice in the control and treated groups after 30 days of oral exposure to Caffeine and Aspartame.

b. Histopathological examination of the ovarian section of *Mus musculus*

The histopathological evaluation of ovarian sections revealed marked differences between the control and experimental groups in both the exposure periods. The ovary of female mice of the control group showed normal histoarchitecture including normal follicles, different sizes and stages of developing oocyte, zona granulosa and thecal layers of follicle and corpus luteum. However, the female mice exposed to Caffeine and Aspartame for 15 and 30 days showed histopathological changes in the ovarian structure characterized by decreased number of growing follicles, hemorrhagic distortions, cellular degeneration, and increased interstitial spaces indicative of tissue damage. Degenerating oocytes, theca layers, corona radiata and zona granulosa were also seen (Fig 4.10).

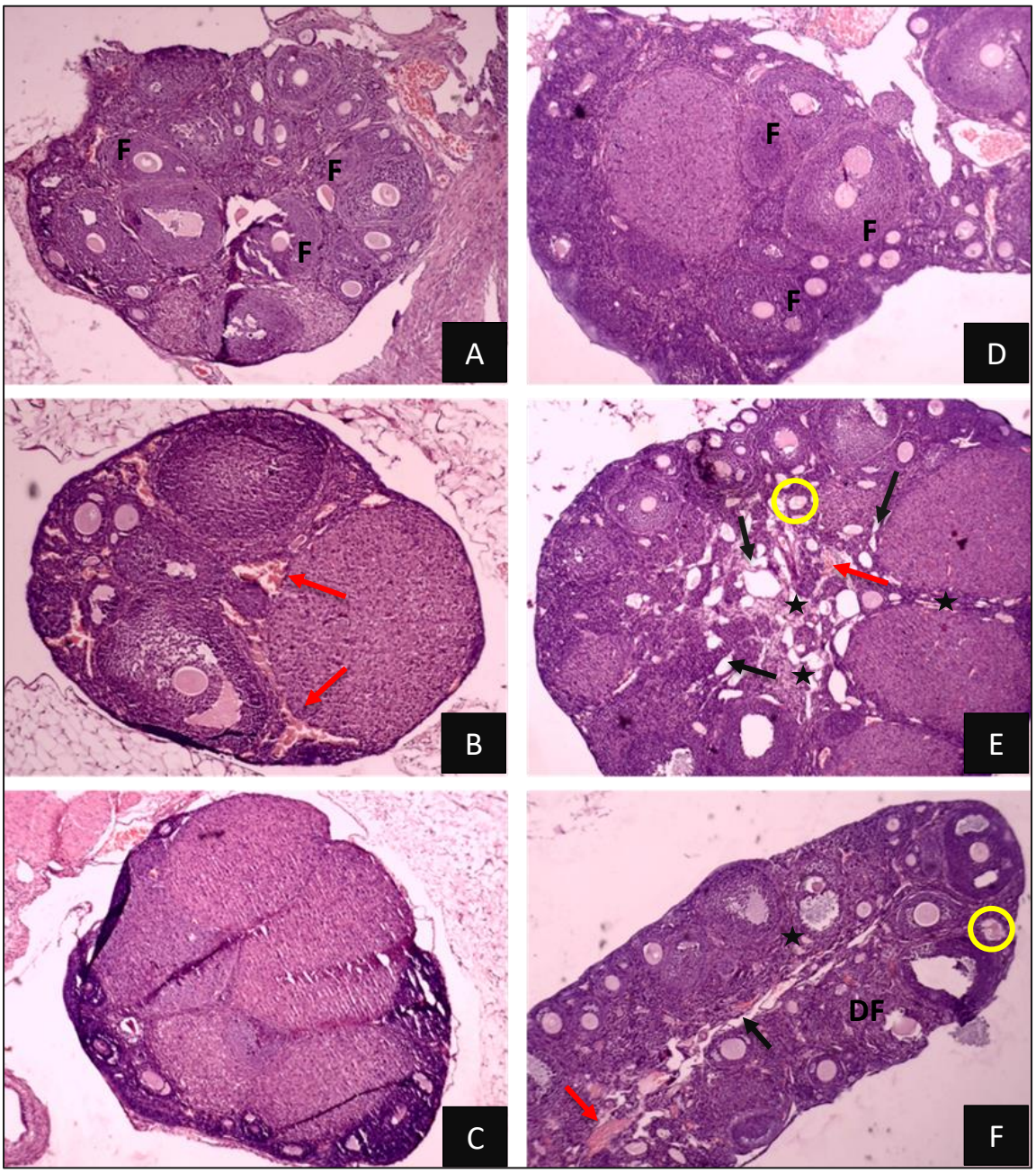

Figure 4.10 Representative images showing histological changes in ovarian sections of mice in control and treated groups after 15 days and 30 days of oral exposure to Caffeine and Aspartame.

For 15-day exposure: (A) Section of ovary of control mice showing normal growing follicles (F) of different sizes and stages and normal histoarchitecture. **(B)** Section of ovary of mice treated with a lower dose of caffeine and aspartame (Exp. 1) showing decreased number of growing follicles and hemorrhagic distortions were observed (red arrow). **(C)** Section of ovary of mice treated with a higher dose of caffeine and aspartame (Exp. 2) showing a great decrease

in the number of growing follicles. Degenerative changes were observed in follicular structure (DF).

For 30-day exposure: (D) Section of ovary of control mice showing normal growing follicles (F) of different sizes and stages and normal histoarchitecture. **(E)** Section of ovary of mice treated with a lower dose of caffeine and aspartame (Exp. 1) showing decreased number of growing follicles and hemorrhagic distortions were observed (red arrow). A lot of cellular degeneration (star) and interstitial spaces were also seen (black arrow). Degeneration of oocyte was also observed (circle). **(F)** Section of ovary of mice treated with a higher dose of caffeine and aspartame (Exp. 2) showing notable histopathological changes. These included a decreased number of growing follicles, hemorrhagic distortions (indicated by red arrows), extensive cellular degeneration (marked with a star), and increased interstitial spaces (highlighted by black arrows). Additionally, degeneration of oocytes and follicles was evident.

4.2 Discussion

Body weight

During the 15-day exposure period, the control group showed a significant increase in body weight, while Experimental groups 1 and 2 exhibited significant decreases in body weight. This suggests that exposure to caffeine and aspartame led to weight loss in the experimental groups compared to the control group. The findings are consistent with previous research indicating that caffeine and aspartame may have effects on body weight (Presti, 2011; Fahey & Treasure, 1991).

In contrast, over the 30-day exposure period, the control group showed only a slight increase in body weight, while experimental groups 1 and 2 experienced significant decreases in body weight. This indicates that the effects of caffeine and aspartame on body weight may become more pronounced over a longer exposure period. The observed decrease in body weight in the

experimental groups is consistent with the results of previous studies (Murray, 1988; Fahey & Treasure, 1991). According to Fahey & Treasure, 1991 caffeine has appetite suppressant, diuretic and stimulant effects which could have led to weight loss. The reduction in body weight may also be due to reduced food and water intake which may have been caused by ASP consumption. These observations are supported by Rolls (1991) who reported that ASP intake induces satiety, decreases food intake and bodyweight.

Thus, the findings suggest that both caffeine and aspartame may have a significant impact on body weight, with longer exposure periods leading to more pronounced effects.

Ovary weight

During the 15-day exposure period, there was a decrease in the average ovary weight in Experimental groups 1 and 2 compared to the Control group. However, statistical analysis using one-way ANOVA test did not reveal a significant difference in ovary weights among the three groups. This suggests that the exposure period and doses of caffeine and aspartame did not have a significant effect on ovary weight within this timeframe.

After a 30-day exposure period, a significant difference in ovary weights among the three groups was observed. Experiment group 2 had the lowest ovary weight, followed by experiment group 1, with the control group having the highest ovary weight. This suggests that prolonged exposure to caffeine and aspartame resulted in a decrease in ovary weight, with higher doses potentially leading to more pronounced effects.

Uterus Weight

During the 15-day exposure period, there was a decrease in the average uterus weight in experimental groups 1 and 2 compared to the control group. However, statistical analysis using the one-way ANOVA test did not reveal a significant difference in uterus weights among the three groups. This suggests that the exposure period did not have a significant effect on uterus weight within this timeframe.

After a 30-day exposure period, a significant difference in uterus weights among the three groups was observed. Experimental group 2 had the lowest uterus weight, followed by Experimental group 1, with the control group having the highest uterus weight. This indicates that prolonged exposure to caffeine and aspartame resulted in a decrease in uterus weight, with higher doses potentially leading to more pronounced effects.

It was reported that aspartame can act as chemical stressor by increasing corticosteroid level which in turn has been shown to decrease the size and weight of organs due to oxidative damage (Choudhary & Devi, 2015). A study conducted by Naik et.al (2023) also revealed similar results with regards to ovary weight and uterus weight.

Gonadosomatic Index (GSI)

During the 15-day exposure period, there was a decline in the average GSI of Experimental groups 1 and 2 compared to the Control group. However, one-way ANOVA analysis revealed no significant difference in GSI among the Control, Experimental 1, and Experimental 2 groups, indicating that the exposure period did not have a significant effect on GSI within this timeframe.

After the 30-day exposure period, significant differences in GSI were observed among the Control, Experimental 1, and Experimental 2 groups. The control group exhibited the highest GSI, followed by Experimental group 1, and Experimental group 2 had the lowest GSI. This suggests that prolonged exposure to caffeine and aspartame resulted in alterations in GSI, with higher doses potentially leading to more pronounced effects. Naik et.al (2023) also found similar results.

Estrous Cycle

During the 15-day exposure period, mice in the control group completed an average of 3 estrous cycles, with all four stages (Proestrus, Estrous, Metestrus, and Diestrus) observed. Experimental group 1, exposed to a low dose of caffeine and aspartame, completed a similar average of 3 cycles. However, Experimental group 2, subjected to a higher dose, completed slightly fewer

cycles, averaging 2.75 cycles. The most prevalent stages observed in the control and experimental groups were metestrus and estrus, with a shift towards estrus in the experimental groups. Initially synchronized, the estrous cycles across all groups showed changes in cycle duration and stage frequencies over time, indicating potential effects of caffeine and aspartame exposure.

Over the 30-day exposure period, the control group completed an average of 5 cycles, with all four stages observed. Experimental Group 1 completed an average of 4.75 cycles, and Experimental Group 2 completed 4.5 cycles. Diestrus was the predominant stage in the control and Experimental Group 1, while estrus and diestrus were more frequent in Experimental Group 2. Similar to the 15-day period, the cycles initially synchronized but became desynchronized over time, suggesting potential effects of prolonged exposure.

During both exposure periods, mice in Experimental groups 1 and 2, treated with caffeine and aspartame, exhibited a decrease in follicle count compared to the Control groups. In the 15-day period, a notable reduction in follicles, particularly in Experiment group 2, suggests a potential negative impact of the substances on ovarian folliculogenesis. This trend persisted in the 30-day exposure period, indicating a prolonged effect on follicle development. A study by Gilbert and Rice (1991) revealed that Caffeine inhibits the action of adenosine, which in laboratory studies affects LH and FSH, which could in turn have an affect on menstrual cycle length.

Follicle Count

Regarding specific follicle types, significant differences were observed among the groups. In the 15-day period, Experimental groups 1 and 2 showed a significant decrease in primordial follicles compared to the Control group. Experimental group 2 had the lowest count of primordial follicles. No significant difference was observed in the number of primary follicles. However, a significant difference was found in late primary follicles, with Experimental group 2 exhibiting the lowest count. No significant difference was observed in secondary and graafian follicles.

Similarly, in the 30-day period, Experimental group 2 had the lowest count of primordial follicles, followed by Experimental group 1. No significant difference was observed in the number of primary follicles, but a significant difference was found in late primary follicles, with Experimental group 2 showing the lowest count. No significant difference was observed in secondary follicles, but there was a significant difference in graafian follicles.

Ovarian Histopathology

The histopathological examination of ovarian sections provided insights into the structural changes induced by exposure to caffeine and aspartame in Mus musculus. In the control group, the ovaries exhibited normal histoarchitecture, characterized by the presence of various follicles at different developmental stages, including developing oocytes, zona granulosa, and thecal layers. Additionally, the presence of corpus luteum indicated normal ovarian function. Conversely, mice exposed to caffeine and aspartame for both 15 and 30 days displayed histopathological alterations in ovarian structure. These changes included a decrease in the number of growing follicles, indicative of impaired folliculogenesis. Moreover, observations of haemorrhagic distortions, cellular degeneration, and increased interstitial spaces suggested tissue damage within the ovarian tissue.

Exposure to caffeine and aspartame, two commonly consumed substances in various food and beverage products, can have significant implications for ovarian health and reproductive function in female mammals. Understanding the mechanisms underlying their effects requires a comprehensive exploration of their impact on hormonal regulation, epigenetic modifications, gut microbiota, neurotransmitter systems, mitochondrial function, and histopathological changes within ovarian tissue.

Chronic consumption of caffeine and aspartame may disrupt hormonal regulation by interfering with key signalling pathways involved in metabolism and reproduction. Caffeine, a central nervous system stimulant, may modulate insulin signalling and glucose metabolism, leading to dysregulation of ovarian function. Aspartame, an artificial sweetener, may influence the release

of gonadotropin-releasing hormone (GnRH), a crucial regulator of the hypothalamic-pituitary-gonadal (HPG) axis. Dysregulation of these pathways might result in altered follicular development, impaired oocyte maturation, and disturbed steroidogenesis, ultimately affecting reproductive health. Furthermore, exposure to caffeine and aspartame can induce epigenetic modifications that alter gene expression patterns in ovarian cells. DNA methylation and histone modifications, influenced by environmental factors such as diet and chemical exposure, may lead to changes in gene expression profiles involved in follicular growth and development. These epigenetic changes may have long-lasting effects on ovarian function and fertility. Gut microbiota composition is another factor influenced by caffeine and aspartame consumption. These substances can alter the balance of beneficial and harmful bacteria in the gut, leading to the production of metabolites that may affect systemic inflammation and immune function. Dysbiosis of the gut microbiota can contribute to hormonal imbalances and inflammatory responses that impact ovarian health. In addition to hormonal and metabolic effects, caffeine and aspartame might have influenced neurotransmitter systems in the brain, affecting neuroendocrine signalling pathways involved in reproductive function. Caffeine, for example, can modulate the release of neurotransmitters such as dopamine and serotonin, which play roles in regulating mood, appetite, and reproductive behaviour. Disruption of these neurotransmitter systems can indirectly impact ovarian function through effects on the HPG axis. Mitochondrial dysfunction within ovarian cells represents another potential mechanism by which caffeine and aspartame may affect reproductive health. Mitochondria are the primary sites of energy production in cells and are essential for processes such as oocyte maturation and steroidogenesis. Exposure to caffeine and aspartame might impair mitochondrial function, leading to reduced energy production, increased oxidative stress, and cellular damage within ovarian tissue.

Histopathological examination of ovarian tissue from animals exposed to caffeine and aspartame reveals structural changes indicative of reproductive dysfunction. Decreased follicle counts, haemorrhagic distortions, cellular degeneration, and increased interstitial spaces are

commonly observed in these animals. These histological alterations provide further evidence of the deleterious effects of caffeine and aspartame on ovarian health and function.

Collectively, these mechanisms contribute seem to menstrual cycle disturbances, ovulatory dysfunction, and reduced fertility in female mammals exposed to caffeine and aspartame. Chronic consumption of these substances may increase the risk of subfertility and reproductive disorders, with potential long-term consequences for reproductive health.

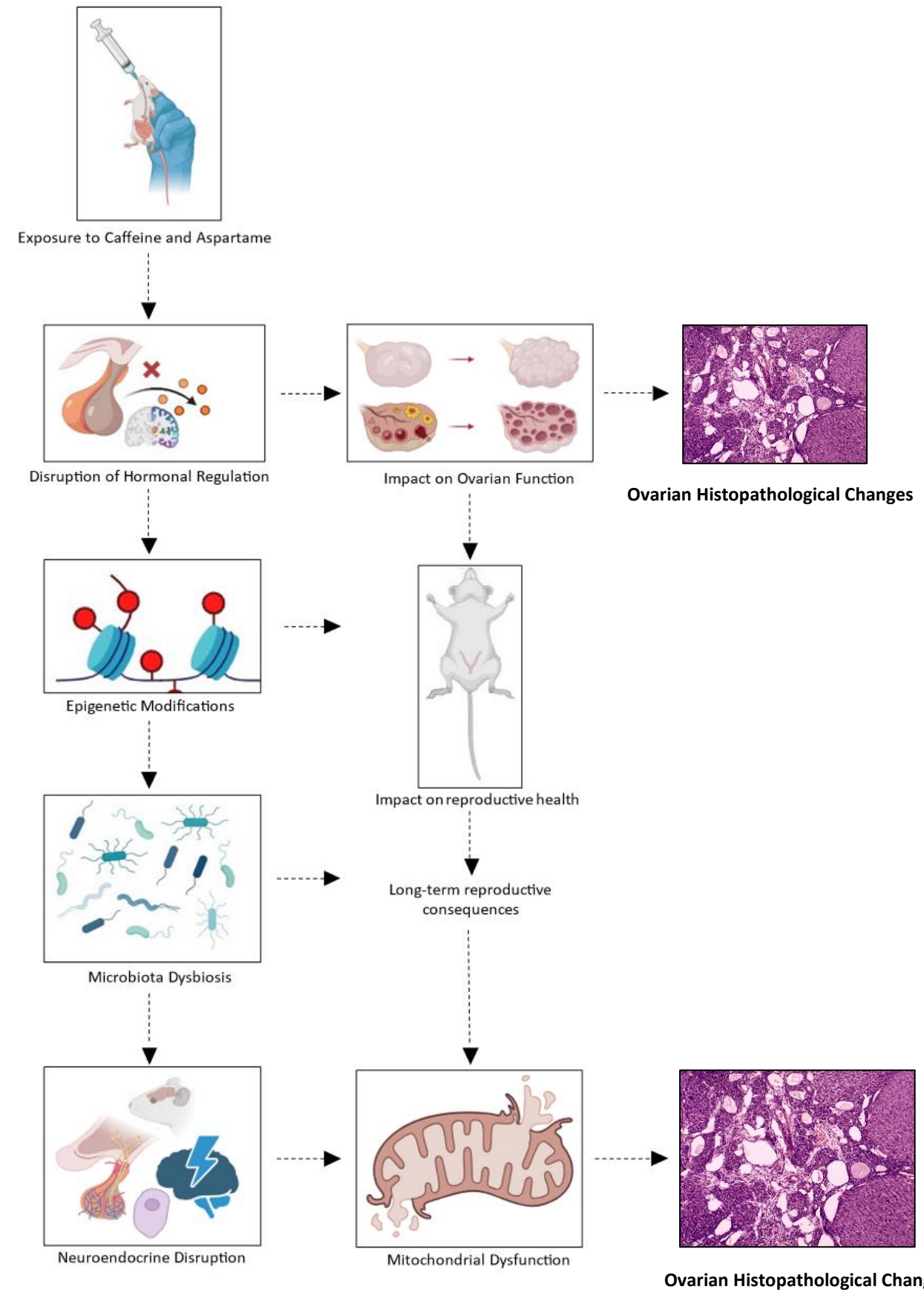

Figure 4.11 Possible Mechanism of Action of Caffeine and Aspartame on Mouse Ovary

4.3 Conclusions

The study investigated the effects of caffeine and aspartame exposure on ovarian health, reproductive function, and estrous cycle length in Mus musculus. Through a comprehensive analysis of body weight changes, organ weights (ovary and uterus), gonadosomatic index (GSI), estrous cycle length, follicle counts, and histopathological examination, several key findings emerged:

Body Weight Changes: Caffeine and aspartame exposure resulted in significant alterations in body weight, with varying effects observed over 15-day and 30-day exposure periods. While the control group exhibited weight gain, experimental groups showed weight loss, suggesting a potential impact of these substances on metabolism and energy balance.

Organ Weight Alterations: Exposure to caffeine and aspartame led to changes in ovary and uterus weights, particularly after the 30-day exposure period. Experimental group 2 showed the most pronounced decrease in both organ weights compared to control and Experimental group 1, indicating a potential adverse effect on reproductive organs.

Gonadosomatic Index (GSI): The GSI, a measure of ovarian health relative to body weight, decreased significantly in experimental groups after the 30-day exposure period, suggesting impaired ovarian function and reproductive health.

Estrous Cycle Length: Caffeine and aspartame exposure influenced the length and synchronization of estrous cycles, with experimental groups showing alterations compared to the control group. These changes indicate potential disruptions in reproductive hormone regulation and ovarian function.

Follicle Counts: Histological analysis revealed a decrease in follicle counts, particularly in Experimental group 2, suggesting a negative impact on ovarian folliculogenesis and oocyte development.

Histopathological Changes: Examination of ovarian tissue showed histopathological alterations, including haemorrhagic distortions, cellular degeneration, and decreased follicle numbers, indicative of tissue damage and impaired ovarian function.

Mechanism of toxicity: The observed toxicity of caffeine and aspartame appears to result from a complex interplay of hormonal, metabolic, oxidative, and inflammatory pathways, highlighting the multifactorial nature of reproductive toxicity induced by environmental exposures.

Need for future investigations: Future research should delve into the intricate molecular mechanisms driving the toxicity of caffeine and aspartame on ovarian health and reproductive function, particularly focusing on hormonal, metabolic, oxidative, and inflammatory pathways. Investigating the long-term repercussions of exposure and understanding the synergistic effects of these substances on reproductive health is also crucial. This knowledge will advance our understanding of environmental factors affecting female fertility and may guide strategies to safeguard reproductive health in humans and wildlife.

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