EFFECTS OF ASPARTAME ON TRANSFORMING GROWTH FACTOR BETA-INDUCED FACTOR 2 LIKE, X-LINKED (TGIF2LX) IN MALE SPRAGUE DAWLEY RATS

BY

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CERTIFICATION

We the undersigned hereby certify that **ABBE-OSAWARU ARESE** with matriculation number **BMS1802328** carried out this work, in the department of medical Biochemistry, University of Benin, Benin city and we approve same as adequate in scope and quality for the award of Bachelors of Science of science degree (B.sc) in Medical Biochemistry.

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(EXTERNAL EXAMINER) **DATE**

DEDICATION

I dedicate this research work to God Almighty for his love, favour and protection upon me and also to my parents, Mr. and Mrs. Osawaru for their care and support towards my education.

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ABSTRACT

Aspartame, an artificial non-saccharide sweetener is commonly used as a sugar substitute in foods and beverages. Aspartame has been reported to negatively influence spermatogenesis; a process in which Transforming Growth Factor Beta-Induced Factor 2 Like, X-Linked (TGIF2LX) gene is required for normal cellular proliferation levels and for differentiation processes. The aim of this study was to investigate the effect of aspartame on Transforming Growth Factor Beta-Induced Factor 2 Like, X-Linked (TGIF2LX) in male Sprague Dawley rats, for this study, 31 pre-pubertal male Sprague-Dawley rats were grouped into 5. Group 1 received 0.5ml distilled water, while aspartame was administered orally via gastric gavage at 40 mg/kg, 80 mg/kg, 160 mg/kg, and 320 mg/kg body weight to group 2, 3, 4, and 5 respectively for 75 days. The results showed a dose dependent decrease was observed in the relative expression of the TGIF2LX gene amongst the groups administered aspartame, starting from 40 mg/kg aspartame to 160 mg/kg aspartame. In conclusion, the decreased expression might be an indication that aspartame can suppress normal cellular proliferation levels and for differentiation processes rather than stimulate them.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Artificial Sweeteners (non-nutritive sweeteners) are sugar substitutes which are several times sweeter than normal sugar (Poshala, 2020). Among these sugar substitutes, aspartame stands out for its intense sweetness and wide use in sugar-free and diet products: it has been used as a sweetening agent in more than 6000 different type of food products like dessert mixtures, chewable multi-vitamins, frozen desserts, and table top sweeteners (Ali et al*.,* 2017).

Aspartame has been extensively studied for their effects on health, including their impact on metabolic processes. There is growing interest in understanding their potential influences on specific genes related to health and reproduction (Ali *et al*., 2021).

One gene important in reproductive process is the Transforming Growth Factor beta-induced Factor 2 like, X-linked (TGIF2LX). TGIF2LX is located on X chromosome, it is homologous with a region on the Y chromosome, and is expressed in the testicular tissue (E Silva, 2018).

TGIF2LX is required for normal cellular proliferation levels and for differentiation processes during spermatogenesis and also believed to be involved in various cellular processes including gene regulation and cell growth (Freitas, 2018).

1.2 AIM OF STUDY

We aimed to understand if the consumption of aspartame has any noteworthy impact on the levels of expression of TGIF2LX in the testes of these rats. This is crucial as significant changes in TGIF2LX as a result of aspartame exposure, may have broader implications for human health, particularly in individuals who frequently consume products containing aspartame.

1.3 JUSTIFICATION OF THE RESEARCH

Aspartame is a widely used artificial sweetener, and there are concerns about its potential effect on male reproductive health. Since TGIF2LX is a key player in various cellular processes related to growth and development, any changes in its levels or functioning may have far-reaching consequences for male reproductive health. By investigating its impact on TGIF2LX, we hope to provide valuable information on the relationship between aspartame and TGIF2LX expression in the testis; this is essential for ensuring the well-being of individuals who rely on these artificial sweeteners.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 SWEETENERS

Sweeteners or sugar alternatives are sugar substitutes that duplicate the effect of sugar in taste with less food energy. Some sugar substitutes are natural, and some are synthetic (Tanu and Kiran, 2015). Sweeteners are additives which provide the basic taste of sweetness to a food product (Neacsu and Madar, 2014).

2.1.1 NATURAL SWEETENERS

Natural sweeteners are sweet-tasting substances that are derived from natural sources, such as fruits, plants, and vegetables (Mazur and Rembiałkowska, 2015).

Examples of natural sweeteners:

1. Honey: Honey is a natural sweetener produced by bees from the nectar of flowers. It contains natural sugars like fructose and glucose and is used as a sweetener, condiment, and in various natural remedies (Bogdanov *et al.,* 2008).
2. Maple Syrup: Maple syrup is derived from the sap of maple trees, primarily in North America. It is a natural sweetener often used as a topping for pancakes and waffles (Seeram *et al.,* 2008).
3. Stevia: Stevia is a natural sweetener derived from the leaves of the stevia plant. Stevia leaves contain steviol glycosides including stevioside, rebaudioside, steviolbioside, and

isosteviol, which are responsible for the plant's sweet taste, and have commercial value all over the world as a sugar substitute in foods, beverages and medicines (Abbas *et al.,* 2017).

4. Date Syrup: Date syrup is made from dates and is a natural sweetener used in Middle Eastern and North African cuisines. It is rich in natural sugars and has a distinct caramel-like flavor (Vayalil, 2002).
5. Agave syrup: (AS), also referred to as agave nectar, is a recently developed food product made from agave plant sap, particularly *Agave salmiana* and *Agave tequilana*, that is, salmiana and blue agave, respectively. Given its low glycemic index and vegan status, this product has become popular as a substitute for traditional sweeteners such as table sugar (sucrose) and honey (Thalheimer, 2015).

2.1.2 ARTIFICIAL SWEETENERS

They are synthetically produced substitutes for sugar whose sweetening power is significantly higher per unit of weight (up to 4000 times sweeter, depending on the type) than sucrose (Dhartiben and Aparnathi, 2017).

They have very few or no calories and are not carbohydrates (Saraiva *et al.,* 2020). Artificial sweeteners are produced from extracts of plants or by safe chemicals (Wani and Bhat, 2019).

Artificial sweeteners, sometimes known as sugar substitutes, are chemicals that are added to various foods and beverages to make them taste sweeter (Choudhary *et al.,* 2015).

Aside Aspartame, four other artificial sweeteners have been approved by the The Food and Drug Administration (FDA):

a) **Saccharin:** Saccharin is the oldest and first artificial sweetener. Its acceptable daily intake (ADI) is lowest among the four sweeteners according to the formulation of the World Health Organization (WHO) (Ali *et al.,* 2021). It is used in products like carbonated and noncarbonated beverages, dairy products, table top sweeteners, juice, jams, chewing gum, confectionaries, desserts, puddings and jellies (Weihrauch and Diehl, 2004).

b) **Sucralose:** Sucralose is 600 times as sweet as sugar with no calories (Gujral *et al.,* 2021). In spite of being produced from sucrose, it is non-nutritive because of its poor absorption in the body (Ali *et al.,* 2021). Sucralose is a stable molecule due to its hydrophilic nature, limited metabolism of the fraction absorbed, rapid elimination and lack of bio accumulative potential (Rocha *et al.,* 2016). It is used as a sweetening agent in bakery items. Carbonated beverages, dairy product, desserts, jams, pie fillings, juices, frozen desserts, other processed food products and gelatine also contain sucralose (Ali *et al.,* 2021). In view of the fact that sucralose remains undigested in our body, it is excreted in the faeces without any modifications (Ali *et al.,* 2021).

c) **Acesulfame-K:** It is potassium salt of 6-methyl-1, 2, 3-axathiazine-4 (3H)-one 2, 2-dioxide with molecular formula C4H4KNO4S and molecular weight of 201 (Marinovich *et al.,* 2013). It is a white crystalline powder, soluble in water. It is a high intensity sweetener, 200 times as sweet as sucrose and devoid of calories. It is heat stable; hence, can be used in cooking and baking. Due to its stability, it is used as a sweetening agent in frozen desserts, baked foods, candies, beverages, cough drops, and beverages (Ali *et al.,* 2021). It may have a bitter after taste when used by itself, so to get rid of it, Ace-K is often combined with other sweeteners like sucralose or aspartame, wherein a synergistic

effect is displayed, one sweetener balances the other after taste making the amalgamation sweeter than its individual constituents (Ali *et al.,* 2021).

d) **Neotame:** Neotame is a derivative of aspartame produced by N-alkylating aspartame. Depending on the kind of food and composition of its blend, its degree of sweetness varies. (Ali *et al.,* 2021). It is 7000 to 13,000 times and about 30 to 60 times sweeter than sugar and aspartame respectively, and has no calories (Ali *et al.,* 2021). Since its approval by the FDA in 2002, it serves as a sweetener in beverages, baked foods, gelatines, chewing gum, jams, jellies, and many other foodstuffs as flavour enhancer. Neotame is an odourless, white to grey-white powder. It is freely soluble in alcohols and slightly soluble in water. The 0.5% aqueous solution of neotame is weakly acidic having pH 5.8 (Whitehouse, 2008).

2.2 ASPARTAME

Aspartame is a dipeptide of the amino acids aspartic acid and phenylalanine joined by a methyl ester (L-aspartyl-L phenylalanine methyl ester). It is marketed as Candere, Equal and Nutrasweet. It cannot be utilised in cooking and baking as it is not heat stable (Ali *et al.,* 2021).

Owing to its clean and good sweet taste profile, aspartame has been used as a sweetening agent in more than 6000 different type of food products like dessert mixtures, chewable multi-vitamins, frozen desserts, and table top sweeteners (Ali *et al.,* 2017).

2.2.1 CHEMICAL AND PHYSICAL PROPERTIES OF ASPARTAME

Aspartame is composed of two amino acid L-aspartic acid and L-phenylalanine. It is a white crystalline, odourless and intensively sweet powder with molecular formula C14H18N2O5 along with the molar mass 294.31 g mol−1. The density of Aspartame is 1.347g/cm3; with a high melting point between 246-247°C (Singh *et al.,* 2019).

Under strong alkaline and acidic conditions aspartame becomes hydrolysed; in aqueous solution, the relationship between pH and stability of aspartame is a bell-shaped curve with the maximum stability at pH 4.3. The solubility of aspartame changes in respect of changes in pH and temperature. Aspartame is also slightly soluble in alcohol; but more soluble in higher temperature acidic solutions (Zafar *et al.,* 2017). Being a dipeptide, aspartame has some limitations as it loses its integrity during heating, therefore cannot be used in baking, cooking or prolonged storage in liquids. It is slightly soluble in water. There is a liner relationship of solubility of aspartame with the temperature and pH. Aspartame has maximum stability at pH 4.3 (Zafar *et al.,* 2017).

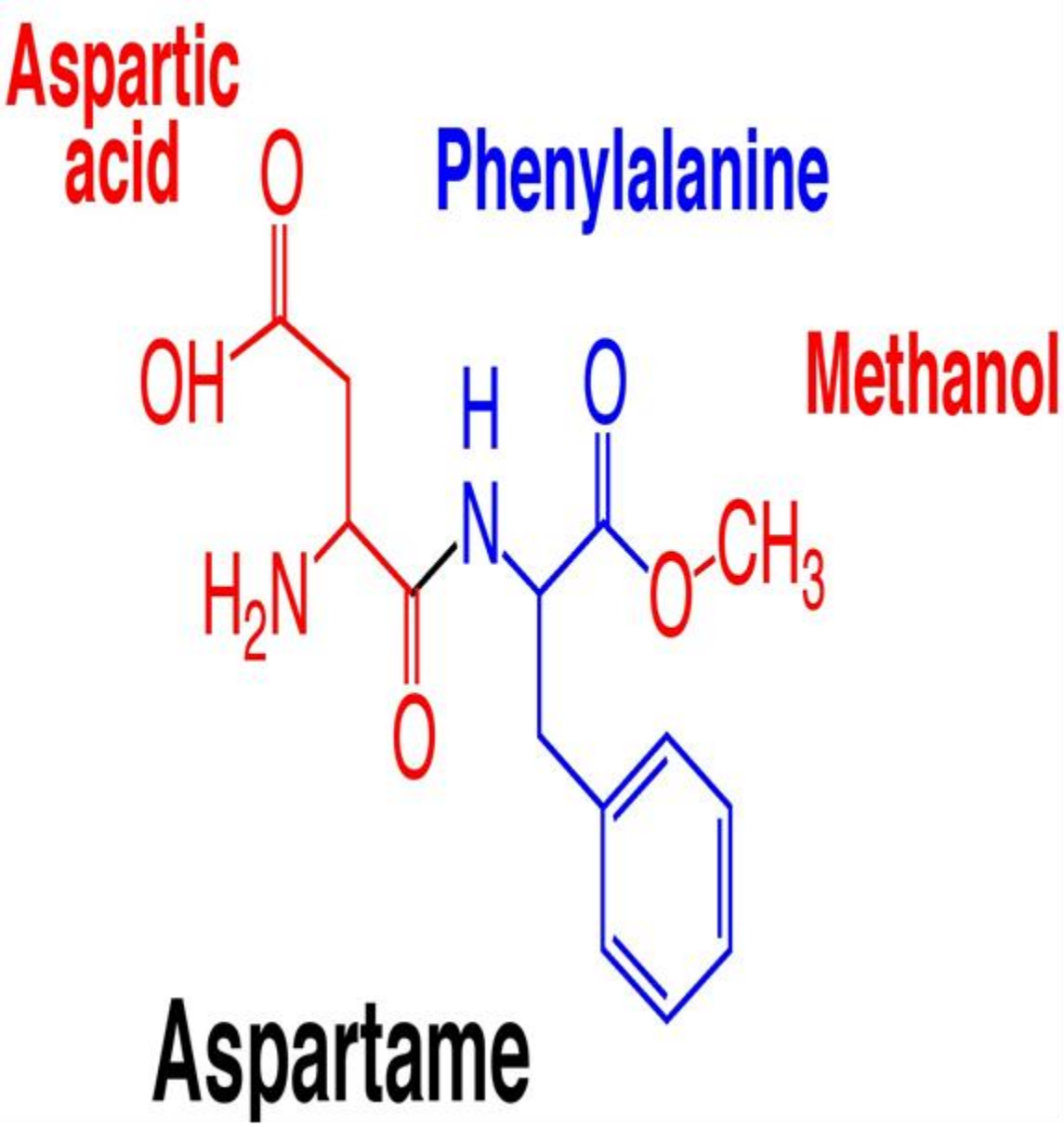

Figure 2.1: Structure of Aspartame

Source: Saeed and Mustafa (2020).

2.2.2 SYNTHESIS OF ASPARTAME

The two major processes for chemical synthesis of aspartame are referred to as Z- and F-processes depending on the presence of the protecting group on the aspartyl moiety. Along with formation of the anticipated EDIT-aspartame, some undesirable ß-coupled products are also produced in the process (Ali *et al.,* 2021).

In the Z-process, benzyloxy carbonyl-L-aspartic acid is dehydrated using acetic anhydride; the resultant dehydrated product is then fixed with the methyl ester of L-phenylalanine in toluene giving a mixture of benzyloxy carbonyl α and β aspartames. Further, the process of hydrogenolysis eliminates the protecting groups yielding a mixture of aspartame isomer, which in turn, on crystallization give off aspartame (Farag *et al.,* 2022).

The synthesis of aspartame by F-process protects the amino group of aspartic acid with a formyl group and is simultaneously dehydrated to form anhydride. Subsequently, the formed anhydride is further treated either with L-phenylalanine or its methyl ester and the protecting groups are eliminated by acid hydrolysis; later on, the resultanant mixture of α and β products are esterified with aqueous methanol which then undergoes crystallization followed by neutralization to yield aspartame (Ali *et al.,* 2021).

2.2.3 METABOLISM OF ASPARTAME

Ones ingested, aspartame undergoes hydrolysis in the intestinal lumen and converts into three hydrolytic products named as phenylalanine (50%), aspartic acid (40%) and methanol (10%) (Humphries *et al.,* 2008); it further breaks down to form formaldehyde, formic acid, and diketopiperazine which further metabolizes in vivo (George *et al.,* 2010). However, methanol

production is not very high during aspartame metabolism but still; it contributes to the toxicities (Heber, 2004).

Production of essential amino acid phenylalanine is a health hazard to those born with phenylketonuria (PKU) - a rare inherited disease (Zafar *et al.,* 2017).

2.2.4 TOXICITY OF ASPARTAME

Methanol, the third component of aspartame is a potentially harmful metabolite (Messih, 2015). A 330 mL can of soft drink sweetened with 550mg/L of aspartame theoretically generate 18.3 mg of methanol, approximately 0.26mg/kg in a 70kg (154lb) person (Messih, 2015). The methanol produced from aspartame is excreted from the body in the same way as methanol produced from other sources such as bananas or tomato juice (O'Donnell, 2012). People born with PKU; a rare inherited disease need to be cautious while consuming products containing aspartame as an ingredient because one of the major constituents of aspartame is phenylalanine (Abhilash *et al.,* 2011). The consumption of aspartame at higher doses may lead to hepatocellular injury and affects the liver antioxidant grade by causing changes in the glutathione dependent system (Abhilash *et al.,* 2011). Mitochondria damage by aspartame, promotes cell apoptosis leading to production of GABA; after that, cell wall disruption occurs and cells become more permeable leading to damage of cellular endothelium of the capillaries (Zafer *et al.,* 2017). Aspartame gives rise to oxidative stress resulting in neuro-degeneration. Direct and indirect neurological side effects related to consumption of aspartame also adversely affect learning and emotional functioning. It alters the concentrations of norepinephrine, epinephrine and dopamine. Lack of sleep, seizures, depression, and headaches are the possible side effects of alteration in regional brain concentrations of catecholamines (Humphries *et al.,* 2008).

2.2.5 EFFECTS OF ASPARTAME ON MALE FERTILITY

Evidence indicates that oxidative stress can cause sperm abnormalities through various mechanisms such as inducing lipid peroxidation in sperm plasma membrane, sperm motility disorder, sperm abnormal morphology and fracture in sperm DNA. Also, literature shows that sperm DNA damage caused by oxidative stress increases apoptosis in immature sex cells leading to decreases in the concentration of sperm (Anbara et al., 2020). Earlier studies showed that aspartame reduced sperm count, viability and motility in rats, which are in accordance with the findings of this study related to decreases in sperm viability and motility following administration of medium- and high-dose aspartame (Ashok et al. 2017). The mechanism of action of aspartame may also be mediated via its effect on Leydig cells: With degradation and atrophy of Leydig cells under the influence of formaldehyde produced from aspartame, the levels of synthesis and secretion of testosterone decreases (Hozayen et al., 2014), which perfectly matched with findings which presented a significant decrease in serum testosterone level in the high-dose group of aspartame (Anbara *et al.,* 2020).

2.3 THE TESTES

The internal structures of the male reproductive system are the testes, epididymis, vas deferens, and prostate, and the exterior structures are the scrotum and penis (Gurung *et al.,* 2022). These structures are well-vascularized and contain numerous glands and ducts that support sperm generation, storage and ejaculation; for fertilization, as well as the production of key androgens for male growth (Tiwana and Leslie, 2023).

Being the principal and vital organ in the male reproductive system, the testis has three main functions namely: produces spermatozoa, synthesizes testosterone--male sex hormone and participate with the hypothalamus-pituitary unit in regulating reproductive function. Regulation of testicular function involves a feedback loop in which the secretion of pituitary gonadotropins which stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus and modulated by testicular hormones (Preston *et al.,* 2012). In addition to affecting fertility, the testicular function includes a central role in the expression of numerous sexually selected traits such as musculature, aggression and sexual behaviour (Preston *et al.,* 2012).

The term spermatogenesis describes and includes all the processes involved in the production of gametes, whereas steroidogenesis refers to the enzymatic reactions leading to the production of male steroid hormones. Spermatogenesis and steroidogenesis take place in two compartments morphologically and functionally distinguishable from each other. These are the tubular compartment, consisting of the seminiferous tubules and the interstitial compartment between the seminiferous tubules (Ilacqua *et al.,* 2018).

2.3.1 CELLS OF THE INTERSTITIAL COMPARTMENT

The most important cells of this compartment are the Leydig cells. Aside from Leydig cells, the interstitial compartment also contains immune cells, blood and lymph vessels, nerves, fibroblasts and loose connective tissue.

a. Leydig Cells: These cells were first described in 1850 by Franz Leydig (1821–1908). Leydig cells produce and secrete the most important male sexual hormone, testosterone (Zirkin and Papadopoulos, 2018). From the developmental, morphological and functional viewpoint, different types of cells can be distinguished: stem Leydig cells as founder cell, progenitor Leydig cells as a committed stem cell, fetal Leydig cells as a terminally differentiated cell in the fetus, and adult Leydig cells as the terminally differentiated Leydig cell (Ge and Hardy, 2007).

b. Macrophages. Lymphocytes and Nerve Fibers: For every 10–50 Leydig cells, one macrophage is to be found. The macrophages probably influence the function of the Leydig cells, in particular their proliferation, differentiation and steroid production, through the secretion of cytokines (Heinrich and DeFalco, 2020). Macrophages secrete stimulators and inhibitors of steroidogenesis. Pro-inflammatory cytokines, reactive oxygen species, nitric oxide and prostaglandins can inhibit Leydig cell function (Hales, 2007).

2.3.2 CELLS OF THE TUBULAR COMPARTMENT

a) Spermatogenesis takes place in the tubular compartment. This compartment represents about 60–80% of the total testicular volume. It contains the germ cells and two different types of somatic cells, the peritubular cells and the Sertoli cells (Bhushan *et al.,* 2016). The testis is divided by septa of connective tissue into about 250–300 lobules, each one containing 1–3 highly convoluted seminiferous tubules (Weinbauer *et al.,* 2010).

b) **Peritubular cells:** The seminiferous tubules are covered by a lamina propria, which consists of a basal membrane, a layer of collagen and the peritubular cells (myofi broblasts); these cells are stratified around the tubulus and form up to concentrical layers that are separated by collagen layers. They also secrete extracellular matrix and factors typically expressed by connective tissue cells: collagen, laminin, vimentin, fibronectin, growth factors, fibroblast protein and adhesion molecules (Albrecht *et al.,* 2006; Schell *et al.,* 2008). Myofibroblasts are poorly differentiated myocytes with the capacity of spontaneous contraction. Peritubular contractility is mediated by endothelin and this effect is modulated by the relaxant peptide adrenomedullin produced by Sertoli cells (Romano *et al.,* 2005). Mice with selective peritubular cell androgen receptor deficiency, revealed defects in contractility-related genes, e.g., e endothelin-1 and endothelin receptor A and B, adrenomedullin receptor and vasopressin receptor 1a (Zhang *et al.,* 2006).

c) **Sertoli cells:** Sertoli cells synthesize and secrete a large variety of factors: proteins, cytokines, growth factors, opioids, steroids, prostaglandins, modulators of cell division etc. Another important function of Sertoli cells is that they are responsible for final testicular volume and sperm production in the adult, with each individual sertoli cell being in morphological and functional contact with a defined number of sperm (Weinbauer *et al.,* 2010).

   Sertoli cells have built tight junctions between each other, the so-called blood-testis-barrier. Lack of connexin-43, a predominant gap-junction protein prevents Sertoli cell maturation associated with continued division of Sertoli cells, and spermatogenic arrest beyond spermatogonial development (Qazilbash *et al.,* 2007; Maherali *et al.,* 2007).

Expression of Sertoli cell markers such as transferrin, androgen-binding protein and junctional proteins such as N-cadherin, connexin-43, gelsolin, laminin-γ3, occludin, testin, nectin, zyxin and vinculin is androgen-dependent (Zhang *et al.,* 2006). It appears that several of these components are involved in establishing the blood testis-barrier, but also in the release of sperm and subsequent remodelling of the Sertoli cell-germ cell junctions (Yan *et al.,* 2008).

d) Germinal Cells: Spermatogenesis starts with the division of stem cells and ends with the formation of mature sperm. The various germ cells are arranged in typical cellular associations within the seminiferous tubules known as spermatogenic stages and the entire spermatogenic process can be divided into four phases:
- Mitotic proliferation and differentiation of diploid germ cells (spermatogonia) (spermatogoniogenesis)
- Meiotic division of tetraploid germ cells (spermatocytes) result in haploid germ cells.
- Transformation of spermatids into testicular sperm (spermiogenesis).
- Release of sperm from the germinal epithelium into the tubular lumen (spermiation) (Weinbauer *et al.,* 2010).

2.4 GENETIC CONCEPT

Human genome is composed of 23 pairs of nuclear chromosomes, 22 pairs are autosomes and one pair of sex chromosome (XX in female and XY in male), with a body's cells being classified as: somatic cells and germ cells (gametes). While somatic cells (non-reproductive cells) are diploid (46 chromosome) and forms the basic units in organ tissues, germ cells (eggs and sperm) are haploid 23 chromosomes and only present in the ovaries and testes respectively. In addition, human genome includes mitochondrial chromosomes where each mitochondrion contains

multiple copies of a small chromosome. Mitochondrial chromosomes are of maternal origin because they are entirely inherited from the cytoplasm of the fertilized ovum (Hamada *et al.,* 2012).

Chromosomes are composed of chromatin which is a complex of unbroken long, double stranded and tightly wound DNA which carries the genes and proteins which help in packing chromosomes in the nucleus. DNA wrapped around histones forms a nucleosome, which is the basic subunit of chromatin. Single DNA strand is composed of simple units called nucleotides which are essentially formed of nitrogenous bases (adenine, guanine, cytosine and thymine; abbreviated A, G, C and T), sugar (deoxyribose) and phosphate, the two DNA strands are interconnected through specific hydrogen bonds between specific nitrogenous bases forming base pairs (Hamada *et al.,* 2012).

Genes are stretches of DNA sequence that encode specific functions such as synthesis of protein through mRNA transcription or synthesis of functional RNA (Hamada *et al.,* 2012).

2.5 GENES AFFECTING FERTILITY

Currently, genetic diseases contribute to 15–30% causes of male infertility (Ferlin *et al.,* 2007). Male factor infertility is responsible for more than 50% of cases of infertility (Jarow *et al.,* 2002); and more than 50% of these cases are of unknown origin; genetics may partly or entirely conduce to the problem of infertility in such men (Dohle *et al.,* 2010).

Phenotypic characteristics of infertile men are immense including impaired spermatogenesis, reduction of testicular size, hypogonadism and sperm dysfunction; however, as aforementioned, the currently known genetic diseases contribute to less than 15–30% of male infertility and not all these phenotypic abnormalities have been unravelled (Dohle *et al.,* 2010).

A large percentage of human male infertility is estimated to be caused by mutations in genes involved in primary or secondary spermatogenesis and sperm quality and function; many of which are located on the Y chromosome and some on the X-chromosome (Stouffs *et al.,* 2009).

2.5.1 Y CHROMOSOME LINKED GENES

The Y chromosome is an obvious area of interest in the study of male factor infertility because it contains many of the genes that are critical for spermatogenesis and the development of male gonads. The variation present on the Y chromosome and the occurrence of deletions of large segments of the chromosome involving multiple genes make it difficult to determine the exact cause of certain infertile phenotypes (Reynolds, 2005). Furthermore, the same phenotype may be produced by several different deletions or mutations. This fact complicates efforts to distinctively correlate mutations with infertile phenotypes. Y chromosome microdeletions are a frequent cause of infertility in males (Katherine *et al*., 2010).

A microdeletion is defined as a chromosomal deletion that spans several genes but is not large enough to be detected using conventional cytogenetic methods (Schlegel, 2004.). Studies have revealed that microdeletions are more prevalent in men who are azoospermic and severely oligozoospermic (Katagiri *et al.,* 2004). The prevalence of microdeletions in azoospermic men was found to range from 10%–15% (Dohle *et al*., 2002). In oligozoospermic men, the prevalence of microdeletions was 5%–10% (Foresta *et al.,* 2001).

Microdeletions most frequently occur on the long arm of the Y chromosome, Yq, and deletions in this region are specifically related to failure of spermatogenesis (Skaletsky *et al*., 2003). A particular area of interest on Yq is the azoospermia factor region (AZF region), which contains

genes involved in the growth and development of sperm (Katherine *et al*., 2010). The AZF region contains three subregions: AZFa, AZFb, and AZFc (Vogt, 2005). The most common aberrations that occur in the AZF region are multiple gene deletions in the AZFb and AZFc areas which can produce a wide range of infertile phenotypes (Ferlin *et al.,* 2007). Microdeletions in the AZF region are most often found in azoospermic and oligozoospermic men with normal karyotypes (Vogt, 2005). Researchers are attempting to characterize deletions in the AZF region so that they can be used to determine treatment for infertile males (Vogt, 2005).

2.5.2 X-LINKED GENES

Many X-linked genes are expressed in the testis (Wang *et al.,* 2001), and are thought to be involved in gametogenesis. The androgen receptor (AR) gene is located on the long arm of the X chromosome (Nuti and Krausz, 2008). It plays a role in meiosis and the conversion of spermatocytes to round spermatids during spermatogenesis (De Gendt *et al.,* 2004). A recent study of infertile men determined that approximately 2% had mutations in their AR gene, while the control population had none (Ferlin *et al*., 2006). Mutations of the AR gene can also lead to androgen insensitivity syndrome (Ferlin *et al., 200*7); this result from mutations that impede the ability of androgens to bind to their receptor, and from decreased transactivation potential (Nuti and Krausz, 2008).

Kallmann syndrome (KS) is another genetic condition that can cause infertility in males and has both X-linked and autosomal genetic components. KS is defined as idiopathic hypogonadotropic hypogonadism (IHH) combined with anosmia or hyposmia; this disorder is caused by a defect in the migration of the GnRH neurons (Bhagavath and Layman*,* 2007). IHH is characterized by low levels of sex steroids in combination with low to normal levels of FSH and LH (Katherine *et al.,*

2010). Patients can either be afflicted with complete or incomplete IHH, which leads to a range of stages of sexual development (Katherine *et al.,* 2010). The absence or low levels of sex steroids inhibit or stunt sexual development and spermatogenesis in males. In addition to reduced sexual development, KS patients have cognitive impairments, ocular abnormalities, midfacial clefting, and renal agenesis (Katherine *et al.,* 2010). Two genetic deletions found to be specifically related to KS patients are KAL1 (KS 1 sequence) and FGFR1 (fibroblast growth factor receptor 1) (Maglott *et al.,* 2005; Bhagavath *et al.,* 2007). KAL1, located on the short arm of the X chromosome, is involved in the migration of the GnRH neurons, and it codes for anosmin-1, a cell adhesion molecule (Bhagavath *et al.,* 2007). KAL1 mutations are thought to be responsible for 30%–70% of KS in patients. Deletion of the FGFR1 gene on chromosome 8 can cause either anosmic or hyposmic forms of KS; this variation in phenotype indicates the reduced penetrance of the deletion within the population (Bhagavath *et al.,* 2007). The discovery of the causes of KS is clinically relevant because it can help clinicians and researchers to understand the proper development of the neuroendocrine axis and regulation of pubarche (Katherine *et al.,* 2010).

Klinefelter syndrome is one of the most frequent cytogenetic anomalies found in infertile men (Gakas *et al.,* 2001). The most frequent type of karyotype present in men with Klinefelter syndrome is 47, XXY The syndrome can also be related to mosaicism (Gakas *et al.,* 2001), higher number of X chromosomes such as 48, XXXY; 48, XXYY or even 49, XXXXY and structurally abnormalities in sex chromosomes (Bojesen *et al.,* 2003). Notably, Klinefelter syndrome men present hypogonadism, azoospermia, small testes, erectile dysfunction and higher gonadotropin levels compared to normal and fertile men (Bonomi *et al.,* 2017). Patients with XX male syndrome (46, XX) are less common than Klinefelter syndrome (Vorona *et al.,* 2007).

Uneven crossing over between X and Y chromosomes may result in an additional X chromosome bearing the SRY gene through a translocation process (Rigola *et al.,* 2002). Patients with XX male syndrome are infertile and may develop male external genitalia, micropenis, hypospadia and cryptorchidism (Vorona *et al.,* 2007). Klinefelter syndrome is easily detected through conventional cytogenetic analysis but XX male syndrome requires molecular cytogenetic with SRY probe to be performed (Katherine *et al.,* 2010).

The Homeobox genes are a diverse group of genes characterized by a conserved 180 base-pair motif encoding a homeodomain with three structurally conserved helices (Hu *et al.,* 2011). These genes have a particular DNA sequence, the homeobox which encodes a very variable protein domain named homeodomain. Many homeodomain proteins are transcription factors with important roles in embryonic development and cell differentiation, several of which are involved in human disease and congenital abnormalities (Holland *et al.,* 2007; Reza *et al.,* 2013).

2.6 TGIF2LX GENE

TGIF2LX (transforming growth factor beta-induced factor 2 like, X-linked) is located on X chromosome; it is homologous with a region on the Y chromosome and is expressed in the testicular tissue (E Silva, 2018). TGIF2LX is required for normal cellular proliferation levels and for differentiation processes during spermatogenesis (Freitas and Silva, 2018).

The human TGIF2LX gene is located on X chromosome (Xq21.3) and is suggested to have originated from the retrotransposition of autosomal TGIF2 (E Silva, 2018). The TGIF2LX has 2 exons with a 96 base-pair intron. Although TGIF2 and TGIF2LX show extensive variations, they share conservationally within the homeodomain, and the C-terminus region (Blanco-Arias *et al.,*

2002). This gene is altered in patients with Turner syndrome and may also be related to severe cases of infertility (Stouffs and Lissens, 2012).

A number of results implicate a potential role of human TGIF2 or TGIF2LX in the regulation of cell growth. Previous study also suggested that TGIF2LX could be involved in negative regulation of cell cycle (Glenisson *et al.,* 2007; Raoofian *et al.,* 2013). Nevertheless, only a few functional studies of human TGIF2LX have been reported and underlying mechanisms remain to be addressed (Raoofian *et al.,* 2013).

One subfamily of homeobox genes encodes proteins with atypical homeodomain referred to as Three Amino-acid Loop Extension (TALE) which are described by the presence of three extra amino acids between helix 1 and helix 2 (Holland *et al.,* 2007). The third helix has a major role in DNA-binding site recognition. TALE homeodomain proteins are crucial transcription factors for embryonic progress and early development (Melhuish *et al.,* 2001; Powers *et al.,* 2010). Four TALE classes have been identified in animals: PBC, MEIS, TGIF and IRO (Iroquois) (E Silva, 2018). TGIF (referred to as transforming growth factor-βinduced factor 5'-TG-3' interacting factor) genes encode TALE -class homeodomain proteins including TGIF1, TGIF2, TGIF2LX (transforming growth factor-βinduced factor 2-like, X-linked) and TGIF2LY (transforming growth factor-β-induced factor 2-like, Y-linked), that act as multifunctional repressors of signalling; these transcription factors function in normal and abnormal developments (Holland *et al.,* 2007). TGIF2 is frequently expressed in human tissues, with especially high expression in the heart, kidney, and testis (Imoto *et al.,* 2000).

It has been shown that TGIF represses gene transcription by acting as a co-repressor of critical co-regulator proteins or signalling transducers for TGF-β signalling pathway (Smads proteins) (Chen *et al*., 2013; Fadakar *et al*., 2016). However, the molecular functions of these proteins

have been predominantly investigated in cultured cells but not routinely in vivo (Hamid and Brandt, 2009); Seo et al., 2006). Recently, a human TGIF paralog protein (named as TGIF2) has been identified that acts as a transcriptional repressor. It has been understood that TGIF2 suppresses transcription by its interaction with histone deacetylases (HDACs) (Ettahar *et al*., 2013; (Hamid and Brandt, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

All chemicals and reagents were of analytical grade. Distilled water was used in all biochemical assays.

1. TRIzol
2. Ethanol
3. Nuclease-free water
4. Chloroform
5. Agarose powder
6. Deoxynucleotide triphosphates (dNTPs)
7. Ethidium bromide (EtBr)
8. Trisboric acid EDTA (TBE)
9. Magnesium Chloride (MgCl2)

10. RNA lysis buffer

3.2 EQUIPMENT

The major equipments used for this study are;

1. Eppendorf Mastercycler (Model: AG 22331 Hamburg)
2. Electronic compact balance (S. METTLER)
3. Sensitive electronic balance (TYPE: LAC214C, 704010)
4. Haier THERMOCOOL CHEST FREEZER (Model: HTF-319H)
5. Centrifuge (Model: D-37520 Osteorode, Kendro laboratory products, Germany)
6. UV- Spectrophotometer
7. Water bath

3.3 ANIMAL AND EXPERIMENTAL PROTOCOL

A total of thirty one (31) male Sprague-Dawley rats were purchased from Animal house of the Department of Biochemistry, Faculty of Life Sciences, University of Benin. The total average weights of the rats when weighed ranged from 80g to 100g and were between three (3) and four weeks (4) old. The animals were housed in a well-ventilated area at TRIGAS Research laboratories at the Department of Medical Biochemistry, University of Benin, in cages floored with sawdust (wood shavings), which was changed and cleaned once every week or when wet, with 12h light and 12h dark cycles. The animals were acclimatized for two weeks before the study started. They were fed with Chukun® grower pellet feed and clean water *ad libitum*. The animals were treated according to the Laboratory Animal Care and Use Guidelines (NAS, 2011). The Faculty of Pharmacy Ethical Committee provided ethical approval for the study, with code,

EC/FP/020/19. After the two-week acclimatization period, the animals were weighed and grouped into cages of five (5) in such a way that the difference in means (weight) of each group is 102 ±2 g. Groups 1 served as the control group and consisted of 6 animals, Groups 2-4 also had six animals each while Group 5 had seven animals.

3.4 PREPARATION AND ADMINISTRATION OF ASPARTAME

Aspartame was reconstituted appropriately in distilled water and orally administered to the experimental animals via *gavage*. Over the course of the experiment, the weights of the animals were determined weekly using electronic weighing balance and used o moderate the doses of aspartame administered. The experiment (administration) went on for 70 days. On the last day of the experiment, all 30 animals that survived (one died) were fasted overnight, after which the animals were sacrificed on the morning of the following day.

The rats were administered aspartame by gastric *gavage* in the following doses;

Group 1: were fed with feed and 0.5 ml distilled water.

Group 2: 40 mg/kg body weight of Aspartame.

Group 3: 80 mg/kg body weight of Aspartame.

Group 4: 160 mg/kg body weight of Aspartame.

Group 5: 320 mg/kg body weight of Aspartame.

3.5 TISSUE COLLECTION

Animals were sacrificed via cervical dislocation technique after which both testes were harvested, blotted and rinsed in cold normal saline. A portion of the testes was carefully imbedded into 0.03ml TRIzol reagent in an eppendorf tube which was kept in a deep freezer until needed.

3.6 TISSUE HOMOGENISATION

The testis was homogenized in TRIzol reagent. RNA-lysing buffer was added to precipitate the RNA from the solution. It was incubated in a water bath at 37 ^{O}C for 10 minutes, it was then centrifuge at 10,000 RPM for 15 minutes. The supernatant was pipetted using micropipette into a different micro centrifuge tube and stored in a freezer for gene expression analysis.

3.7 GENE EXPRESSION STUDY

3.7.1 ISOLATION OF TOTAL RNA

Total RNA was isolated from tissue samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

3.7.2 cDNA CONVERSION

One (1 μg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs)

in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Elekofehinti *et al.,* 2020).

3.7.3 PCR AMPLIFICATION AND AGAROSE GEL ELECTROPHORESIS

Polymerase chain reaction (PCR) for the amplification of gene of interest was carried out with OneTaqR2X Master Mix (NEB) using the following primers (Inqaba Biotec, Hatfield, South Africa). PCR amplification was performed in a total of 25 µl volume reaction mixture containing cDNA, primer (forward and reverse SEE BELOW) and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95 ◦C for 5 min, followed by 30 cycles of amplification (denaturation at 95 ◦C for 30 s, annealing for 30 s and extension at 72 ◦C for 60 s) and ending with final extension at 72 ◦C for 10 min. The amplicons were resolved on 1.0% agarose gel. The GAPDH gene was used to normalize the relative level of expression of each gene, and quantification of band intensity was done using "image J" software (Olumegbon *et al.,* 2022).

3.7.4 PRIMER SEQUENCES

TGIF2LX-gene

Forward Primer: TCCGTGACTGGCTCCTAA

Reverse Primer: GTCTGCGGGCATTTGTAAAC

3.8 STATISTICAL ANALYSIS

Gene expression analysis was done using the software IMAGEJ. This was used to quantify densitometrically the intensities of the bands from the agarose gel electrophoresis. All data were statistically evaluated and the results was expressed as mean ± standard error of mean (SEM) and analyzed using Graph Pad Prism version 8.0.2 with statistical significance determined by One-way ANOVA (Analysis of Variance).

CHAPTER FOUR

RESULTS

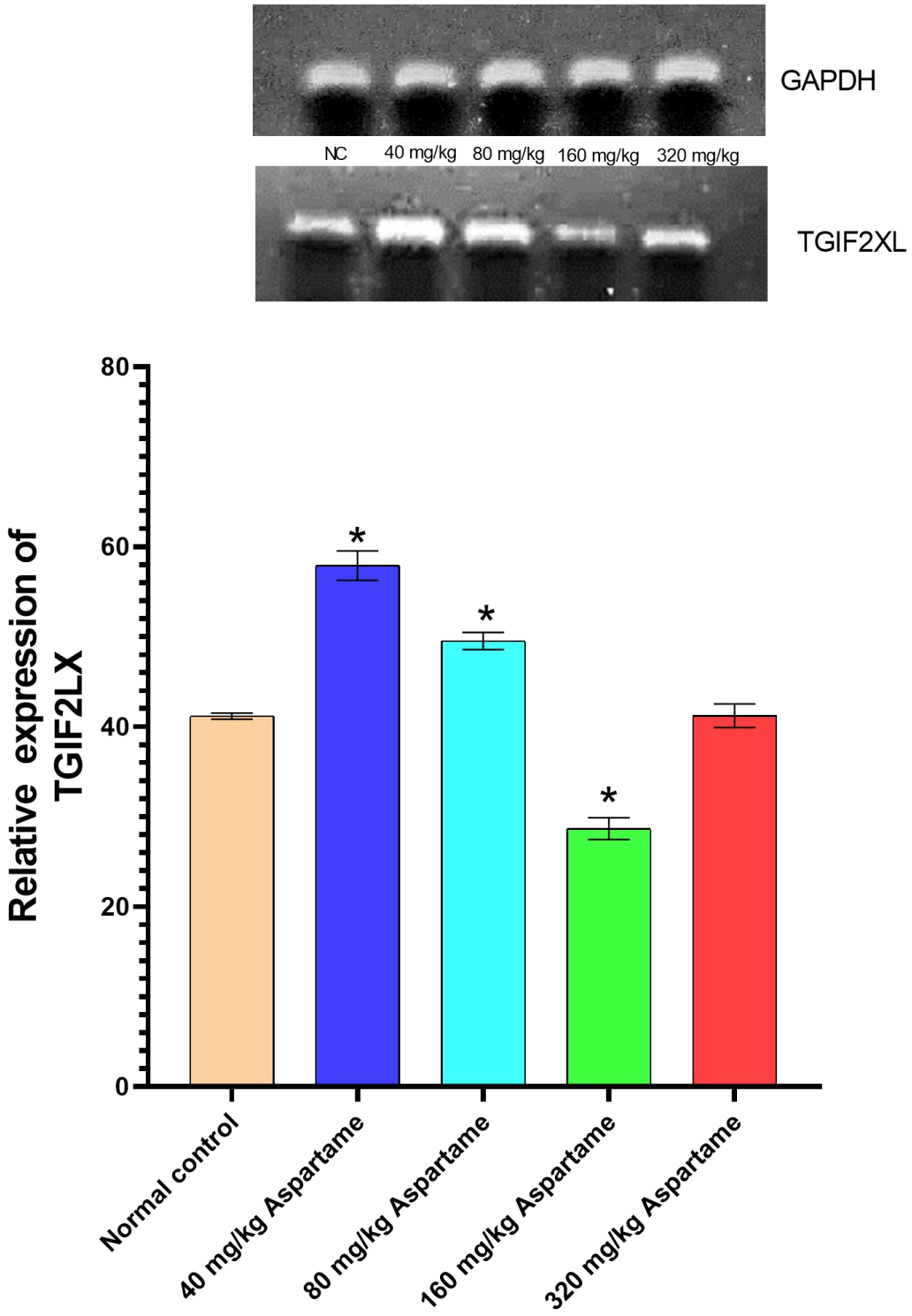

Figure 4.1: Effect of aspartame on Transforming Growth Factor Beta-Induced Factor 2 Like, X-Linked (TGIF2LX) in the testes of male Sprague Dawley rats. * represent significant difference at $P < 0.05$ to normal control.

In comparison to normal control, the group administered aspartame at 320 mg/kg shows that there was no significant expression of TGIF2LX gene. However, there was a significant reduction in expression of TGIF2LX gene in the testis when compared to normal control in the group administered 160 mg/kg of aspartame. Furthermore, 40 mg/kg and 80 mg/kg significantly increased the expression of TGIF2LX gene in the testis when compared to normal control.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Aspartame an artificial non-saccharide sweetener is commonly used as a sugar substitute in foods and beverages (Saraiva *et al.,* 2020). While aspartame is approved as safe for consumption by various regulatory agencies, there have been ongoing debates and studies about its potential effects on health, including male fertility. Some studies have suggested that aspartame may have effects on male fertility genes thereby causing infertility. TGIF2LX gene is required for normal cellular proliferation levels and for differentiation processes during spermatogenesis (Freitas, 2018).

In the study, the findings revealed that gene expression increased significantly at two specific doses, 40 mg/kg and 80 mg/kg of aspartame. However, at higher doses, specifically 160mg/kg, the gene expression decreased, with no significant difference observed at the 320mg/kg dose. The increase in TGIF2LX gene expression at the lower doses (40mg/kg and 80mg/kg) could suggest that at a low dosage of aspartame, TGIF2LX gene is more expressed and may in turn favour male fertility. This is contrary to previous researches done which showed that Aspartame reduces fertility in males (Kearns *et al.,* 2021; Anbara *et al.,* 2021). However, the decrease in gene expression at 160mg/kg may suggest that the TGIF2LX gene expression become repressed at this dose, thereby reducing fertility and agreeing to previous studies conducted that indicated that consumption of aspartame for a long period results in male reproductive toxicity (Kearns *et al.,* 2021; Anbara *et al.,* 2021). Although, groups administered 40 mg/kg, 80 mg/kg and 160 mg/kg was noticed to have a dose dependent decrease in the expression of the TGIF2LX gene, which seems to affirm previous reports suggesting that consumption of aspartame causes a decrease in male fertility (Kearns *et al.,* 2021).

5.2 CONCLUSION

This study shows that the dose dependent decrease that was observed across the groups might be an indication that aspartame can suppress normal cellular proliferation levels and for differentiation processes in the expression of TGIF2LX gene rather than stimulate them. This may imply that long term consumption of aspartame may indeed play a role in male infertility. Although further research is needed to ascertain the effect of aspartame on male fertility genes.

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