POSTNATAL ASSESSMENT OF THE ATTENUATION ACTIVITY OF QUERCETIN AND VITAMIN E ON ACCUTANE-INDUCED HIPPOCAMPAL DAMAGE FOLLOWING PRENATAL EXPOSURE IN WISTAR RATS

BY

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UNIVERSITY OF BENIN
BENIN CITY

NOVEMBER, 2025

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A PROJECT SUBMITTED TO THE DEPARTMENT OF ANATOMY, SCHOOL OF BASIC MEDICAL SCIENCES, COLLEGE OF MEDICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA
IN PARTIAL FUFILMENT OF REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B. Sc) DEGREE IN ANATOMY

SUPERVISED BY
DR (MRS) AKPOROBO EJEGUO

NOVEMBER, 2025

CERTIFICATION

This is to certify that this research work titled "**POSTNATAL ASSESSMENT OF THE ATTENUATION ACTIVITY OF QUERCETIN AND VITAMIN E ON ACCUTANE-INDUCED HIPPOCAMPAL DAMAGE FOLLOWIN PRENATAL EXPOSURE IN WISTAR RATS**" for the award of a degree of Bachelor of Science (B.Sc.) in Anatomy was carried out by **Azubuike Anthonia Chinenyenwa** under the supervision **of Dr. (Mrs.) AKPOROBO EJEGUO**. All literatures used in this study have been acknowledged and properly referenced.

Mrs. AKPOROBO EJEGUO **Date**
Supervisor

Dr. A. B. ENOGIERU **Date**
Head of Department

External Examiner **Date**

DEDICATION

This work is dedicated to the loving memory of my grandmother, **Juliana Ogeri Oko**, whose kindness, strength, and unwavering faith continues to inspire me. Her memory remains a guiding light in all that I do.

ACKNOWLEGMENTS

My sincere and heartfelt gratitude goes first to my mother (**Azubuike Rosemary Ugo)**, for her endless love, prayers, sacrifices, and unwavering support. Her strength and encouragement have been the foundation upon which this work stands. I also appreciate my father (**Azubuike Ogbonnia**) for his guidance and believe in my abilities throughout this journey. Above all, I thank God almighty for his grace, wisdom and direction that sustained me during this research.

I also extend my profound gratitude to my supervisor, **Dr (Mrs.) Akporobo Ejeguo**, for her invaluable love, patience, guidance and constructive criticism during the course of this study. My sincere appreciation goes to **Mr. Sam,** for his immense support during the laboratory bench work, as well as to the **HOD, Dr. A.B. Enogieru**, to my course advisor, **Dr. (Mrs.) R.A. Orheruata**, and all lecturers in the **Department of Anatomy, School of Basic Medical Sciences, University Of Benin**, for their academic contributions and encouragement.

To my siblings, **Azubuike Esther, Azubuike Monica, and Azubuike Samuel**, this wouldn't have been possible without you all. Thank you for your endless love, laughter, care, support and companionship.

Finally, I acknowledge myself, for the resilience, perseverance, and dedication that brought this work to completion.

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ABBREVIATIONS

PND	Postnatal Day
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CRP	C-Reactive Protein
NMDA	N-Methyl-D-Aspartate
DMA	Dimethylacetamide
PTSD	Post-Traumatic-Stress Disorder
EPM	Elevated Plus Maze
g	gram
Mg	milligram
Kg	kilogram

ABSTRACT

Isotretinoin (Accutane), a retinoid widely used for severe acne, has been reported to induce hippocampal toxicity through oxidative stress, apoptosis, and neuroinflammation, which can impair prenatal development. The hippocampus, being particularly vulnerable to oxidative damage, may contribute to learning and memory deficits when exposed to Isotretinoin during gestation. This study evaluates the protective efficacy of Vitamin E, and quercetin against Accutane-induced hippocampal toxicity in prenatal Wistar rats. Four groups were examined in this: control, Accutane only, Accutane + quercetin + vitamin E, and Accutane + vitamin E. Administration was carried out from GD 14-21 of pregnancy. After littering, the neonates were left until PND 31 (postnatal day 31) before sacrifice. Histological analysis of hippocampal integrity, and developmental parameters in offspring (birth weight, neurobehavioral testing) were assessed. Results showed that Accutane exposure led to reduced birth weight and brain weight, along with neuronal degeneration in the hippocampal cells. Co-administration of vitamin E and quercetin significantly improved hippocampal morphology and restored neurobehavioral activity. Neurobehavioral results revealed that Accutane increased transfer latency and reduced exploratory activity, whereas combined antioxidant treatment improved behavioral performance. Offspring from the co-treated group also exhibited higher body weight and brain weights compared to the Accutane-only group. In conclusion, quercetin and vitamin E effectively counteracted Accutane-induced prenatal toxicity in the hippocampus of Wistar rats. The synergistic antioxidant action of both compounds preserved neural architecture and improved developmental outcomes, suggesting their potential as therapeutic agents against retinoid-related neurotoxicity in pregnancy.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Isotretinoin (13-cis retinoic acid) known as Accutane, is a retinoid widely used to treat severe cystic acne (Layton, 2009). Despite its efficacy, isotretinoin is associated with well-established teratogenic effects when administered during pregnancy, including craniofacial, cardiovascular and central nervous system malformations (Lammer *et al*., 1985; Dai *et al*., 2021). Beyond gross congenital anomalies, accumulating evidence suggests that isotretinoin can induce neuropsychiatric and cognitive side effects, with the hippocampus being one of the most vulnerable brain structures (O'Reilly *et al*., 2008).

The hippocampus plays a central role in learning, memory, and emotional regulation, and disruption of its development can have long-lasting consequences on neurocognitive outcomes (Aimone *et al.,* 2014). Studies in rodents and humans have demonstrated that isotretinoin reduces hippocampal cell proliferation and impairs hippocampal-dependent tasks (Crandall *et al.,* 2004; O'Reilly *et al.,* 2008). These toxic effects are thought to be mediated by excessive retinoic acid receptor signaling, oxidative stress, mitochondrial dysfunction, and activation of apoptotic pathways (Wu *et al*., 2016). Since the prenatal brain is particularly sensitive to oxidative and inflammatory stress, isotretinoin exposure during gestation poses a significant risk for abnormal and subsequent neurobehavioral deficits.

Antioxidant therapy has been proposed as a promising strategy to mitigate drug-induced neurotoxicity. Vitamin E (alpha-tocopherol), a fat-soluble antioxidant well known for its ability to scavenge lipid peroxyl radicals, stabilize neuronal membranes, and protect against hippocampal oxidative damage (Traber & Atkinson, 2007). In animal studies, vitamin E supplementation reduced hippocampal lipid peroxidation, preserved synaptic integrity, and improved cognitive performance under conditions of oxidative stress (Rai *et al.,* 2011).

Quercetin, a flavonoid found in fruits and vegetables, also demonstrates neuroprotective properties, including antioxidant, anti-inflammatory, and anti-apoptotic effects (D'Andrea, 2015). Experimental studies have shown that quercetin preserves hippocampal neurons, improves synaptic plasticity, and protects against memory impairment induced by oxidative and inflammatory stress (Kumar & Pandey, 2013). Importantly, quercetin has been shown to complement lipid-phase antioxidants like vitamin E by regenerating oxidized alpha-tocopherol and enhancing overall antioxidant capacity (Boots *et al.,* 2008).

Evidence suggests that combining antioxidants can provide synergistic neuroprotection by targeting different aspects of oxidative and inflammatory cascades (Devi & Muthu, 2015). Thus, co-administration of vitamin E and quercetin may represent a rational strategy to attenuate isotretinoin-induced hippocampal toxicity and improve prenatal neurodevelopmental outcomes.

This study, therefore, aims to evaluate the protective effects of vitamin E and quercetin, both individually and in combination, against isotretinoin-induced hippocampal toxicity and its consequence on prenatal development. By targeting oxidative stress and apoptosis, this approach may offer insight into novel therapeutic strategies to mitigate retinoid-associated neurodevelopmental risks.

1.2 Aim of Study

The primary aim of this study was to investigate the potential neuroprotective effects of vitamin E and quercetin, individually and in combination, against isotretinoin (Accutane)-induced hippocampal toxicity and its subsequent impact on postnatal brain development following prenatal exposure.

1.3 Specific Objectives

1. To evaluate the effect of isotretinoin exposure on hippocampal structure and function during prenatal development.
2. To determine the protective role of vitamin E in mitigating isotretinoin-induced hippocampal toxicity/damage.
3. To assess the protective role of quercetin against isotretinoin-induced hippocampal damage.
4. To investigate whether combined treatment with vitamin E and quercetin exerts synergistic neuroprotective effects against isotretinoin-induced hippocampal injury.
5. To analyze markers of oxidative stress, inflammation, and apoptosis in hippocampal tissue following treatment and interventions.
6. To compare prenatal neurodevelopmental outcomes (structural and functional) between untreated, single-treatment, and combined-treatment groups.

1.4 Statement of Research Problem

1. Accutane (isotretinoin) is effective for acne but linked to hippocampal toxicity and prenatal development risks (O'Reilly *et al*., 2008).

2. These effects raise concern especially in pregnant women.

3. Vitamin E and Quercetin, both antioxidants, may protect against oxidative stress and neuronal injury (Li *et al*., 2016; Traber and Atkinson, 2007).

4. Few studies have explored their combined role in reducing Accutane-induced hippocampal during development.

1.5 Justification of Study

Although isotretinoin is clinically valuable, its teratogenic and neurotoxic effects remain a major concern, particularly during prenatal development. The hippocampus is especially

vulnerable, and damage at this stage can result in long-lasting cognitive and behavioral impairments.

Vitamin E and quercetin are well-documented natural antioxidants with complimentary neuroprotective mechanisms. While each has shown promise in reducing oxidative stress and neuronal damage, the potential synergistic effect of their combined use against Accutane-induced hippocampal toxicity has not been systematically explored.

This study is therefore justified as it addresses a significant gap in knowledge, with potential to provide new insights into antioxidant-based strategies that may mitigate drug-induced neurodevelopmental risks.

CHAPTER TWO

LITERATURE REVIEW

2.1. Drug of Study (ACCUTANE)

Accutane, commonly known by its generic name **Isotretinoin**, is an oral retinoid derived from vitamin A. it was first approved in the early 1980s for the management of severe, recalcitrant nodular acne, a form of acne that doesn't respond adequately to conventional treatment methods such as antibiotics and topical retinoids (Layton, 2009). Its introduction marked a major breakthrough in dermatological therapy, as isotretinoin is capable of inducing long-term remission and, in some cases, permanent resolution of acne (Zaenglein *et al*., 2016).

The drug has been established as the gold standard for severe acne therapy due to its multifaceted action on the key pathogenic factors involved in acne development: increased sebum production, abnormal follicular keratinization, proliferation of *cutibacterium acnes*, and inflammation (Strauss *et al*., 2001). Despite its clinical benefits, isotretinoin has generated significant concern in medical practice because of its broad systemic effects, including teratogenicity, hepatotoxicity, dyslipidemia, and possible neuropsychiatric outcomes (Cunningham *et al*., 2014; Huang & Cheng, 2017). These concerns have made it a focus of continuous pharmacological and toxicological research.

2.1.1 Pharmacology of Accutane (ISOTRETINOIN)

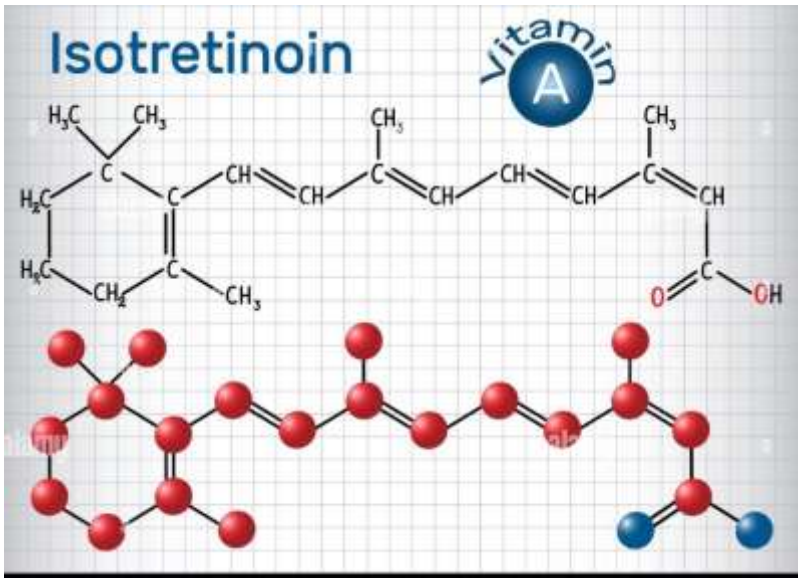


Fig. 2.1: chemical structure of Accutane(isotretinoin) (PubChem (NCBI), 2024).

Isotretinoin is a **13-cis isomer of retinoic acid** that belongs to the retinoid family of compounds. Retinoids are structurally related to vitamin A and exert their biological activity by interacting with nuclear receptors, specifically **retinoic acid receptors (RARs**) and **retinoid X receptors (RXRs)**. These receptors regulate the transcription of genes involved in cellular differentiation, proliferation and apoptosis (Nelson *et al.*, 2013).

The drugs therapeutic efficacy in acne primarily stems from its ability to suppress sebaceous gland activity. Isotretinoin induces apoptosis of sebocytes, leading to a marked reduction in the size of sebaceous glands and a subsequent decrease in sebum secretion up to 90% (Strauss *et al*., 2001). This reduction in sebum removes the lipid-rich environment required for the survival and proliferation of *C. acnes*, a bacterium strongly implicated in acne pathogenesis.

In addition, isotretinoin has significant anti-inflammatory properties. It reduces the expression of toll-like receptors on keratinocytes and monocytes, thereby diminishing inflammatory cytokine production. It also normalizes follicular keratinization, which prevents the formation of microcomedones-the precursors of acne lesions (Zaenglein *et al*., 2016).

Taken together, these mechanisms explain the drug's ability to target multiple pathways in acne pathogenesis, making it uniquely effective compared to other treatments.

2.1.2 Pharmacokinetics

The pharmacokinetic profile of isotretinoin reflects its lipophilic nature. Following oral administration, isotretinoin is absorbed in the gastrointestinal tract, but its absorption is highly variable and significantly enhanced when taken with fatty meals due to its fat solubility. For this reason, administration with food is strongly recommended to improve bioavailability (Layton, 2009).

Once absorbed, isotretinoin is extensively bound to plasma proteins greater than or equal to 99%), primarily albumin, which influences its distribution. The drug undergoes hepatic metabolism, primarily via cytochrome P450 enzymes, to produce several metabolites, including 4-oxo-isotretinoin, which retains some biological activity (Nelson *et al.,* 2013).

Elimination occurs through both renal and fecal pathways, with a reported plasma elimination half-life ranging between 10 to 20 hours. However, its metabolites, especially 4-oxo-isotretinoin, may persist longer in circulation.

2.1.3 Morphology and Production of Accutane (ISOTRETINOIN)

Isotretinoin (13-cis-retinoic acid) is a synthetic derivative of vitamin A (retinol) that belongs to the retinoid class of compounds.

Morphologically, its chemical formula is $C_{20}H_{28}O_2$ and its molecular weight is 300.44g/mol. It has a polyene chain structure with double bonds, terminating in a carboxylic acid group. In its physical state, it is a yellow-orange crystalline powder which is practically insoluble in water but soluble in organic solvents such as ethanol, methanol, and chloroform (Nelson *et al*., 2016). Accutane is light-sensitive and easily oxidized; thus, it is typically stored in opaque, airtight containers under nitrogen to prevent degradation (Layton,2009). This unique

morphology, especially the conjugated polyene system, underlies isotretinoin ability to interact with nuclear receptors, influencing gene transcription and cellular differentiation.

Isotretinoin is not obtained directly from natural sources but is produced semi-synthetically from vitamin A precursors. The production typically begins with beta-ionone, an intermediate derived from the degradation of carotenoids such as beta-carotene. Condensation reactions are used to elongate the carbon chain, forming retinoic acid derivatives. Isomerization is then used to convert all trans-retinoic acid (tretinoin) into its 13-cis isomer (isotretinoin) through controlled chemical processes, often involving heat or catalysts (Blasiak *et al.*, 2019). The crude product is purified via crystallization or chromatography to remove isomeric impurities. Special emphasis is placed on controlling the ratio of cis/trans isomers because isotretinoin's therapeutic efficacy and side effects profile are linked to its configuration (Kang *et al.*, 2005). The final isotretinoin is encapsulated into soft gelatin capsules containing isotretinoin dissolved in soybean oil, hydrogenated vegetable oil, or beeswax, along with butylated hydroxyanisole (BHA) to stabilize the compound.

2.1.4 Physiochemical Properties of Accutane (ISOTRETINOIN)

Table 2.1Physiochemical Properties of Accutane (ISOTRETINOIN)

PROPERTY	**DESCRIPTION**
Chemical name	Isotretinoin (13-cis-retinoic acid)
Molecular formula	$C_{20}H_{28}O_2$.
Molecular weight	300.44g/mol.
Appearance	Yellow to orange crystalline powder.
Solubility	Practically insoluble in water, soluble in ethanol, Methanol, chloroform, and oils.
Melting point	172-175 degree Celsius.
Stability	Light sensitive, oxidizes easily; requires storage in Opaque, airtight containers under nitrogen.
pKa	~4.76 (carboxylic acid group).
Partition Log coefficient (log P)	~6.8 (lipophilic).
Isomeric form	13-cis isomer of retinoic acid.
Pharmacological Class	Retinoid (derivative of vitamin A)

PubChem (2024); DrugBank (2024); Merck Index (2013); FDA (2023)

2.1.5 Therapeutic Benefits of Accutane (ISOTRETINOIN)

Accutane (isotretinoin) is primarily recognized as one of the most effective systemic agents for the management of severe acne. Its therapeutic effects are derived from its ability to modulate sebaceous land function, keratinization, and inflammatory processes. Beyond acne, isotretinoin has also shown benefits in several dermatological and oncological conditions (Nelson *et al.,* 2009; Wu *et a*l., 2020).

2.1.5.1 Severe Nodulocystic and Recalcitrant Acne

~ Isotretinoin is the drug of choice for severe nodulocystic acne and acne that is resistant to conventional therapies such as antibiotics and topical retinoids (Layton, 2009).

~ It induces sebaceous gland involution, reducing sebum production by 80-90%, thereby altering the microenvironment that supports *Cutibacterium acnes* growth (Zouboulis *et al.,* 2014).

~ Clinical remission rates are high, with 70-80% of patients achieving long-term clearance after a single 16–20-week course (Goodfield *et al*., 2010).

2.1.5.2 Prevention of Acne Scarring

~By controlling severe inflammatory acne early, isotretinoin reduces the risk of permanent atrophic and hypertrophic scarring.

~ This therapeutic benefit has significant psychosocial implications, as acne scarring is associated with depression, anxiety, and reduced quality of life. (Keur *et al*., 2020).

2.1.5.3 Other Dermatological Uses

~ Rosacea (severe/ refractory cases): low-dose isotretinoin has shown efficacy in reducing papules, pustules, and erythema in patients unresponsive to standard therapy (Rafa *et al.,* 2013).

~ Seborrheic dermatitis: through its sebosuppresive activity, isotretinoin improves scaling and erythema (Plewig & Jansen 2019).

~ Hidradenitis suppurativa: limited evidence suggests it may help in mid-to-moderate cases by reducing follicular occlusion (Blok *et al.,* 2013).

~ Disorders of keratinization: conditions such as ichthyosis, keratosis follicularis, and palmoplantar keratoderma may benefit from isotretinoin due to its ability to normalize keratinocyte differentiation (Shalita *et al*., 2001).

2.1.5.4 Oncological and Chemoprotective Potential

~ Retinoids, including isotretinoin, have been investigated for their chemoprotective properties against epithelial cancers (Hong *et al.*, 1990).

~ Isotretinoin has been studied in the prevention of squamous cell carcinoma of the skin in high-risk patients (Bavinck *et al*., 1995).

~ Experimental evidence suggests it may inhibit tumor progression by regulating cell proliferation, differentiation and apoptosis.

2.1.5.5 Psychosocial and Quality of Life Improvement

~ Severe acne often leads to depression, anxiety and social withdrawal. Successful treatment with isotretinoin not only improves skin health but also significantly enhances self-esteem, social functioning, and psychological well-being (Rubinow *et al*., 1987).

~ Long-term remission reduces the burden of repeated treatment, improving patients' adherence and confidence in therapy.

Accutane's therapeutic benefits extend far beyond acne clearance-encompassing prevention of scarring, improvement in psychosocial well-being, treatment of other dermatological disorders, and potential chemoprotective applications. Its broad efficacy underscores its place as a cornerstone in dermatological therapeutics.

2.1.6 Accutane and Cardiovascular Risk Factors

Although Accutane (isotretinoin) is primarily prescribed for dermatological conditions, its systemic effects extend beyond the skin. Several studies have investigated its potential influence on cardiovascular risk factors, particularly lipid metabolism, blood pressure, and inflammatory markers.

Hypertriglyceridemia is the most consistent cardiovascular-related adverse effect on isotretinoin therapy. Reports suggest that 25-45% of patients develop elevated triglyceride levels during treatment (Layton, 2009). Severe hypertriglyceridemia (>800-1000 mg/dL) can predispose to acute pancreatitis, a rare but serious complication (Zane *et al*., 2006).

Increases in low-density lipoprotein (LDL-C) and very-low-density lipoprotein (VLDL-C) levels have been observed, while high-density lipoprotein (HDL-C) levels may decrease (Karadag *et al*., 2011).

Isotretinoin may cause elevations in liver enzymes such as ALT and AST in 10-15% of patients (Agarwal & Ramasamy, 2013). Since dyslipidemia and hepatic steatosis are risk factors for cardiovascular disease. These metabolic changes may indirectly enhance long-term cardiovascular risk in susceptible patients.

Current evidence doesn't strongly link isotretinoin to hypertension or significant changes in systemic blood pressure. However, some reports suggest endothelial dysfunction, associated with retinoid therapy, possibly mediated by oxidative stress (Ertugrul *et al*., 2011).

Isotretinoin therapy has been associated with increases in C-reactive protein (CRP), an inflammatory biomarker linked to cardiovascular risk (Karadag *et al.*, 2011). Rare case reports describe venous thromboembolism and myocardial infarction in isotretinoin users, but causality remains uncertain due to confounding factors (Nguyen & Chiou, 2014).

Because isotretinoin can significantly alter lipid metabolism, baseline and periodic monitoring of lipid panels and liver enzymes is strongly recommended during therapy.

Patients with pre-existing cardiovascular disease, metabolic syndrome, diabetes, or family history of dyslipidemia require special caution (Layton, 2009). Lifestyle interventions such as low-fat diet, reduced alcohol intake, and regular exercise can help mitigate isotretinoin-induced dyslipidemia.

Isotretinoin does not directly cause cardiovascular disease, but it modifies key risk factors-especially lipid profile disturbances, mild hepatotoxicity, and inflammatory marker changes. While most effects are reversible upon discontinuation, long-term cardiovascular risk in predisposed patients warrants careful monitoring.

2.2. Antioxidants: Vitamin E and Quercetin

2.2.1.0 Vitamin E

Fig.2.2: Natural Sources of Vitamin E (NIH office of Dietary Supplements, 2024).

Vitamin E is one of the most important lipid-soluble antioxidants in the human body, essential for protecting cells against oxidative stress. It encompasses a family of eight naturally occurring compounds-four tocopherols and four tocotrienols-of which alpha-tocopherol is the most biologically active form in humans (Traber & Atkinson, 2007). Its biological importance lies in its ability to protect cellular membranes, regulate gene expression, and support immune function. Beyond its role in nutrition, Vitamin E has

extensive use in medicine, dermatology, and cardiovascular health research due to its potent antioxidant properties (Niki & Traber, 2012).

2.2.1.1 Morphology and Chemistry of Vitamin E

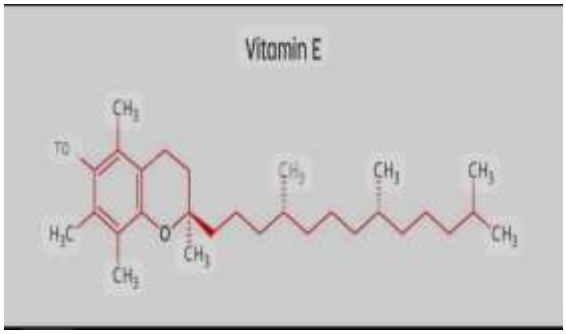


Fig 2.3: chemical structure of Vitamin E (NCBI PubChem, 2024).

Vitamin E consists of a chromanol ring structure with a hydroxyl group that can donate a hydrogen atom to neutralize free radicals, and a long phytyl side chain that allows lipid solubility. This unique structure enables vitamin E to integrate into biological membranes, stabilizing them and preventing oxidative damage (Maret & Traber, 2022). Among the various forms, alpha-tocopherol is selectively transported in plasma and has the highest bioactivity.

2.2.1.2 Physiochemical Properties of Vitamin E

- Molecular formula (alpha-tocopherol): $C_{29}H_{50}O_2$
- Molecular weight: 430.71g/mol
- Appearance: yellow to amber viscous oil
- Solubility: insoluble in water, but soluble in organic solvents such as ethanol, fats and oils.
- Stability: sensitive to oxidation, light, and heat; often stabilized in pharmaceutical formulations with antioxidants such as butylated hydroxyanisole (BHA) (Niki & Traber, 2012).

2.2.1.3 Therapeutic Benefits of Vitamin E

The therapeutic benefits of vitamin E include:

1. Antioxidant defense: Vitamin E neutralizes free radicals and protects polyunsaturated fatty acids in cell membranes from peroxidation (Traber & Atkinson, 2007).
2. Dermatological use: it is widely applied in skincare and dermatology for its role in wound healing, photoprotection, and scar reduction (Thiele *et al*., 2005).
3. Cardiovascular protection: it reduces LDL oxidation, lowering the risk of atherosclerosis and related cardiovascular diseases (Rimm *et al*., 1993).
4. Immune function: Vitamin E enhances immune responses, particularly in elderly populations, by supporting T-cell activity (Meydani *et al*., 2004).
5. Neuroprotective role: Vitamin E may delay the onset or progression of neurodegenerative diseases such as Alzheimer's by reducing oxidative damage in neurons (Maret & Traber, 2002).

2.2.2.0 Quercetin

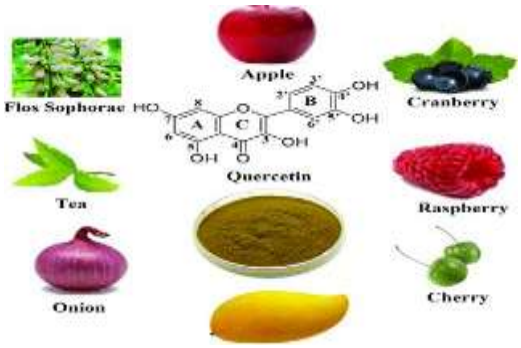


Fig.2.4: sources of quercetin (NIH Office of Dietary supplements, 2024).

Quercetin is a naturally occurring plant flavonoid widely distributed in fruits, vegetables, leaves, and grains, with concentrations found in onions, apples, berries and tea (Li *et al*., 2016). As one of the most abundant dietary flavonoids, it plays a significant role in human nutrition and pharmacology, owing to its strong antioxidant, anti-inflammatory, and cardioprotective properties. Research has shown that Quercetin helps modulate

oxidative stress, supports vascular health, and may provide protective effects against cancer and neurodegenerative diseases (Boots *et al.,* 2008).

2.2.2.1 Morphology and Chemistry of Quercetin

Fig 2.5: chemical structure of quercetin (NCBI PubChem, 2024).

Quercetin belongs to the flavanol subclass of flavonoids. Structurally, it consists of *a three-ring backbone (C6-C3-C6 system)* with multiple hydroxyl substitutions, which are responsible for its strong free radical scavenging and metal-chelating activities (Boots *et al*., 2008).

- Molecular formula: C15H10O7
- Molecular weight: 302.24g/mol
- Appearance: Yellow crystalline powder
- Solubility: poorly soluble in water but soluble in ethanol, methanol, and other organic solvents.
- Stability: sensitive to light and oxidation; stability can be improved by formulation with phospholipids or encapsulation (Li *et al*., 2016).

2.2.2.2 Therapeutic Benefits of Quercetin

The therapeutic benefits of quercetin include the following:

1. Antioxidant activity: Quercetin is a potent free radical scavenger that protects cellular macromolecules from oxidative damage, reducing the risk of chronic diseases (Boots *et al.,* 2008).
2. Anti-inflammatory effects: It modulates pro-inflammatory signaling pathways such as NF-kB and Mitogen-Activated Protein Kinase, thereby reducing the production of inflammatory mediators (Li *et al.,* 2016).
3. Cardiovascular health: Clinical studies indicate Quercetin lowers blood pressure, improves endothelial function, and reduces LDL oxidation, thus reducing cardiovascular risk (Egert *et al*., 2010).
4. Anticancer potential: Quercetin exhibits anti-proliferative effects on cancer cells by inducing apoptosis, arresting cell cycle progression, and modulating key signaling pathways (Khan *et al.,* 2016).
5. Neuroprotection: It may protect neurons from oxidative damage, offering potential in reducing the risk of Alzheimer's and Parkinson's diseases (Spencer, 2009).
6. Immune modulation: Quercetin enhances immune function by regulating cytokine release and strengthening antiviral defense mechanisms (Li *et al*., 2016).

Both Vitamin E and Quercetin share a central role as natural antioxidants that protect biological systems from oxidative damage, but they differ in their source, structure, and mechanisms of action. Vitamin E, being a lipid-soluble vitamin, primarily localizes within cellular membranes where it prevents lipid peroxidation and maintains membrane stability (Traber & Atkinson, 2007). In contrast, Quercetin, a polyphenolic flavonoid, exerts its antioxidant effects through free radical scavenging, metal ion chelation, and modulation of intracellular signaling pathways (Boots *et al*., 2008).

While Vitamin E is most effective in protecting lipophilic environments such as cell membranes and lipoproteins, Quercetin provides broader protection by acting in aqueous and intracellular compartments. Both compounds also exhibit cardioprotective, anti-inflammatory, and neuroprotective properties, making them complementary in their prevention of chronic diseases. Their combined therapeutic relevance highlights the importance of integrating dietary antioxidants into strategies aimed at reducing oxidative stress and promoting human health (Li *et al*., 2016).

2.3. Organ of Study (HIPPOCAMPUS)

The neurological systems of every vertebra animal are centered on the brain (Clifford *et al.*, 2014). The complexity of the brain allows for its involvement in various body functions such as memory, thought control, emotion, motor skills, vision, touch, temperature, breathing, hunger and every process that regulates the body. Together, the brain and the spinal cord that extends from it constitutes the central nervous system (CNS) (Marris, 2018). The average human brain weighs about 1500g (3 lbs.) or approximately 2% of the body weight of a 150-Ib adult, (Roth, 2013). Two broad classes of cells make up the brain, these are the neurons and the glial cells (also known as glia or neuroglia). The neurons however are considered the most important cells in the brain (Kandel *et al*., 2000).

The brain, which is the body's largest cluster of neurons is normally found in the skull, close to organs for special senses such as vision, hearing and smell. The brain, a gelatinous mass is cushioned by the skull, an external capsule of bone, and is invested by a series of three consecutive tissue membranes called meninges (Roth,2013). From outermost to innermost, these meninges are dura matter, arachnoid matter and pia matter. The brain floats within the skull suspended in the cerebrospinal fluid, which acts as a shock absorber and keeps the pressure inside the brain constant (Elizabeth, 2023). The major arteries and vein supplying the brain lie among the meninges (Roth, 2013).

2.3.1 The Hippocampus

The hippocampus is a complex brain structure that is deeply ingrained in the temporal lobe. It plays a significant role in memory and learning. The hippocampal region is the temporal extension of the cerebral cortex (Gilbert and Brushfield, 2009). From the exterior, it is identifiable as a layer of closely spaced neurons that curves into an S-shaped structure on the margin of the temporal lobe. Consequently, it is referred to as a limbic lobe component. Despite being subcortically located, it is not entirely regarded as such. It contains several limbs and is a component of the hippocampus formation (Dhikav 2006 and Dhikav *et al*., 2007). The limbic system is believed to be deep within the brain, a "primitive brain" (Gilbert and Brushfield, 2009). It is centered on an array of issues including appetite, pain, pleasure, mood, sex drive, hunger and memory. The frontal part of the limbic lobe is the amygdala while the posterior part is the hippocampus. According to Papez's (1930) theory, the hippocampus organizes emotional reaction, which is then expressed in the cingulate gyrus by mamillary bodies. It is presently thought to play a role in recalling the past and imagining the future (Addis and Schacter, 2011). Furthermore, studies suggest that this is gradually becoming recognized as a component of the "moral brain" because it is well-known to play a significant role in learning, memory and spatial navigation (Fumagalli and Prori, 2012).

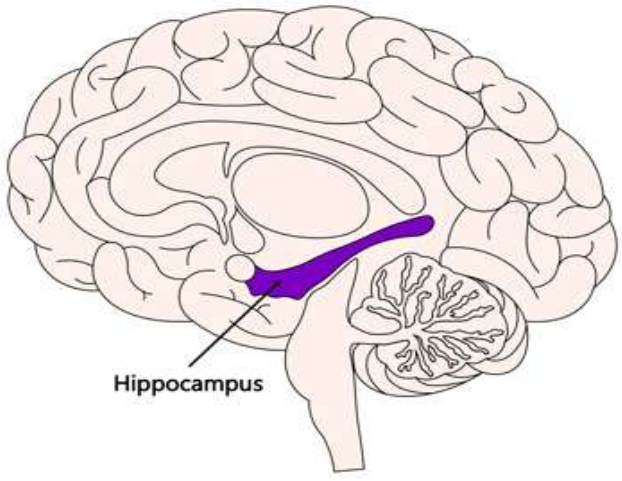


Fig.2.6: The brain showing the hippocampus (Purves *et al.*, 2008).

2.3.1.1 Anatomy of the Hippocampus

The hippocampus is made up of two parts, the dentate gyrus and the cornu ammonis (hippocampus proper). The hippocampal sulcus divides and shapes these two parts into one another. Subiculum is located below the sulcus. The hippocampus is a part of the allocortex (archicortex), therefore, there is a zone that separates it from the neocortex. Some anatomists divide it into the entorhinal region (EC), subiculum, dentate gyrus (DG), and hippocampus proper (cornus ammonis; CA). A complete set is referred to as hippocampal formation (Anand and Dhikav, 2012).

The three parts of the hippocampus are the head, body and tail. The head is the enlarged portion of the hippocampus. Histologically, the hippocampus proper (Cornu ammonis) is divided into CA1, CA2, CA3 and CA4. The subiculum that links the entorhinal cortex with the hippocampal region is situated directly across from the CA1 in the ventricle. The hippocampal region is supplied by the posterior cerebral artery which comprises anterior, middle and posterior branches. The anterior choroidal artery (uncal branch) improves the supply. The hippocampal veins drain into the basal veins. (Anand and Dhikav, 2012).

2.3.1.2 Embrology of the Hippocampus

The hippocampus develops during early embryonic stages and involves neurogenesis, migration, and differentiation (Gupta *et al*., 2020). Its development begins in the ventricular zone where neural stem cells divide to produce progenitor cells called radial glial cells (Gupta *et al*., 2020). These neurons migrate to their final destination within the hippocampus (Dudley *et al*., 2021). This migration process is guided by molecular signals and radial glial cells scaffolding (Das *et al.,* 2019 and Dudley *et al*., 2021). The oldest neurons form the deepest layers of the hippocampus. As the neurons migrate, they form distinct regions in the hippocampus (Dudley *et al*., 2021) these regions which are the dentate gyrus, cornu ammonis

(CA-CA4), and subiculum form the major subdivision of the hippocampal formation (Blaauw and Meiners, 2020).

Once the neurons reach their final locations, they differentiate into different cell types and form distinct layers within the hippocampus (Meng *et al*., 2022). The dentate gyrus develops a characteristic layering pattern, including the granule cell layer and molecular layer (Dudley *et al*., 2021). During later stages of development, the hippocampus establishes connection with other brain regions, such as the entorhinal cortex which sends axons to the dentate gyrus and CA1-CA3 regions forming perforant pathway (Blaauw and Meiners, 2020). These connections are crucial for the integration of information from various brain areas into the hippocampus and the transmission of processed information to other brain regions (Das *et al*., 2019).

2.3.1.3 Histology of the Hippocampus

The hippocampus is a complex structure with distinct cell layers and specialized cell types that contribute to its functions. The dentate gyrus is composed of two layers: the Granule cell layer and the molecular layer. The Granule cell layer houses the densely packed granule cells, which are primarily excitatory neurons of the dentate gyrus (Keshvari *et al*., 2020). The molecular layer contains the dendritic processes, axons, and different types of interneurons (Mandour *et al.*, 2021).

The CA1 regions contains pyramidal neurons arranged in layers, characterized by large, triangular-shaped pyramidal neurons with prominent apical dendrites (de Flores *et al.*, and Piancino et al., 2020). The CA2 region has less distinct cellular characteristics compared to other regions (Piancino *et al*., 2020). CA3 region is characterized by densely packed pyramidal neurons with a prominent triangular shape (Rahmati *et al*., 2022) and is known for its recurrent collateral connections, forming the "auto-associative network" (Lamtai *et al*., 2021).

The CA4 regions is a small transitional area between the dentate gyrus and CA3, containing fewer neurons compared to other regions. The subiculum consists of pyramidal neurons and interneurons. Pyramidal neurons are the principal excitatory cells of the hippocampus, playing a crucial role in the flow of information and connections to other brain regions (de Flores *et al.,* 2020; Mandour *et al*., 2021). The hippocampus also contains various types of inhibitory interneurons which use gamma-aminobutyric acid (GABA) as their principal neurotransmitter (Lamtai *et al.,* 2021; Rashmati *et al*., 2022). Interneurons regulate the excitability and synchronization of hippocampal circuits and contribute to the generation of specific patterns of activity (Rashmati *et al.*, 2022).

2.3.1.4 Layers of the Hippocampus

The hippocampus is a bilateral, seahorse-shaped structure located in the key part of the limbic system. Histologically, it is characterized by a three-layered cortical organization, distinguishing it from the six-layered neocortex (Duvernoy, 2005). The hippocampal formation is composed of the dentate gyrus, hippocampus proper (Cornu Ammonis or CA regions: CA1-CA4), and subiculum.

- Dentate gyrus (DG): it contains granule cells whose axons (mossy fibers) project to CA3 pyramidal neurons.
- Cornu Ammonis (CA) regions:

  ~CA1: this region is predominantly pyramidal neurons, crucial for output to the subiculum and entorhinal cortex.

  ~CA2: it is a smaller region with densely packed pyramidal cells.

  ~CA3: this region receives output from the dentate gyrus; involved in autoassociative memory functions.

  ~CA4 (hilus): it has interneurons that modulate excitatory input from the dentate gyrus.

- Subiculum: it is a transitional area connecting the hippocampus to the entorhinal cortex and other cortical regions.

  The principal neurons (pyramidal and granule cells) are supported by inhibitory interneurons (e.g., basket cells) that regulate excitatory activity. The characteristic trisynaptic circuit-entorhinal cortex-dentate gyrus-CA3-CA1 underlies its role in information processing (Anderson *et al.*, 2007).

2.3.1.5 Functions of the Hippocampus

The functions of the hippocampus are as follows:

1. Memory formation: the hippocampus is essential for consolidation of short-term memory into long-term memory, particularly declarative and episodic memory (Squire, 2009).
2. Spatial navigation: it acts as a cognitive map, with "place cells" in the hippocampus encoding spatial context (O'Keefe and Nadel, 1978).
3. Learning: it is involved in associative learning processes and synaptic plasticity, particularly through long-term potentiation (LTP) in the CA1 region.
4. Emotional regulation: through its connection with the amygdala and prefrontal cortex, the hippocampus participates in stress regulation and emotional memory.
5. Neurogenesis: the dentate gyrus is one of the few brain regions where adult neurogenesis occurs, contributing to learning and memory flexibility (Ming and Song, 2011).

2.4.0 Hippocampal Toxicity

The hippocampus is highly vulnerable to toxic insults due to its rich excitatory circuitry, high metabolic demand, and sensitivity to oxidative stress. Damage to the hippocampus, termed hippocampal toxicity, disrupts its role in memory formation, learning, and emotional

regulation, and is implicated in various neurological and psychiatric disorders (Small *et al.*, 2011).

2.4.1 Mechanisms of Hippocampal Toxicity

1. Excito-toxicity: overactivation of glutamatergic receptors, particularly NMDA receptors, results in excessive calcium influx, leading to neuronal injury and death (Hardingham and Bading, 2010).

2. Oxidative stress: reactive oxygen species (ROS) generated during mitochondrial dysfunction or exposure to neurotoxic agents damage lipids, proteins and DNA in hippocampal neurons (Butterfield and Halliwell, 2019).

3. Neuroinflammation: activation of microglia and release of pro-inflammatory cytokines exacerbate neuronal degeneration (Heneka *et al*., 2015).

4. Apoptosis and Neurodegeneration: toxic insults can trigger intrinsic and extrinsic apoptotic pathways, resulting in neuronal loss, particularly in CA1 and CA3 pyramidal cells (Small *et al*., 2011).

5. Neurogenesis inhibition: environmental toxins, chronic stress, and drugs of abuse reduce neurogenesis in the dentate gyrus, impairing hippocampal plasticity and cognitive flexibility (Ming and Song, 2011).

2.4.1.1 Agents Associated with Hippocampal Toxicty

- Drugs: chemotherapeutic agents (e.g., methotrexate), high-dose corticosteroids, and certain antiepileptics have been linked to hippocampal atrophy and dysfunction (Siegers and Fardell, 2011).
- Environmental toxins: heavy metals such as lead and mercury induce oxidative stress and impair synaptic plasticity.

- Substance abuse: chronic alcohol use and psychostimulants (e.g., methamphetamine) are associated with neuronal damage and reduced hippocampal volume.
- Pathological conditions: neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), epilepsy and chronic stress contribute to hippocampal toxicity through multifactorial mechanisms (Goodman 2012; Moser *et al.*, 2015).

2.4.1.2 Consequences of Hippocampal Toxicity

1. Memory impairment: it leads to deficits in episodic, spatial and working memory.

2. Learning disabilities: it reduces the ability to acquire and retain new information.

3. Mood disorders: it causes anxiety, depression, and stress dysregulation due to hippocampal-amygdala-prefrontal cortex circuit disruption

4. Neurodegenerative progression: hippocampal toxicity accelerates diseases such as Alzheimer's, where hippocampal atrophy is an early hallmark.

2.4.2 Accutane (ISOTRETINOIN) and Prenatal Hippocampal Toxicity

Isotretinoin (13-cis-retinoic acid) is a synthetic derivative of Vitamin A widely used for the treatment of severe nodulocystic acne. Despite its therapeutic effectiveness, Accutane is a well-recognized teratogen and is contraindicated during pregnancy due to its potential to cause severe embryonic and fetal abnormalities. Among the central nervous system (CNS) structures, the hippocampus-a critical brain region involved in learning and memory, and emotional regulation-has been shown to be particularly sensitive.

Prenatal exposure to isotretinoin can disrupt retinoic acid signaling, which is essential for normal neurodevelopment. Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) regulate gene transcription during embryogenesis, guiding neuronal proliferation, differentiation, synaptogenesis, and hippocampal patterning. Excessive isotretinoin interferes

with this tightly regulated pathway, leading to abnormal neuronal migration, impaired synaptic connectivity, and hippocampal malformations (Collins and Mao, 1999; Adams and Lammer, 2012).

Experimental animal studies have demonstrated that isotretinoin administration during pregnancy induces apoptosis in hippocampal neurons, reduces dendritic spine density, and alters hippocampal architecture. These changes manifest functionally as deficits in spatial learning and memory, resembling hippocampal dysfunction syndromes (Ishikawa *et al*., 2008; Crandall *et al*., 2004). In humans, prenatal isotretinoin exposure has been associated with neurodevelopmental disorders, including cognitive impairment, reduced IQ, and learning disabilities, many of which are consistent with hippocampal injury (Lammer *et al*., 1985).

Furthermore, hippocampal toxicity from isotretinoin may involve oxidative stress, excitotoxicity, and mitochondrial dysfunction, which exacerbate neuronal vulnerability during critical stages of brain development. This neurotoxicity is compounded by the fact that the hippocampus undergoes significant growth and synaptic organization during the second and third trimesters of gestation, making it particularly susceptible to teratogenic insults (Maden, 2007).

In summary, Accutane (isotretinoin) exerts profound teratogenic effects on the developing hippocampus when administered prenatally. By disrupting retinoid signaling and inducing neurotoxic cascades, isotretinoin leads to structural and functional hippocampal abnormalities that underlie long-term cognitive and behavioral impairments. This underscores the strict contraindication of isotretinoin in pregnancy and highlights the importance of understanding its neurotoxic mechanisms in prenatal brain development.

2.5. Isotretinoin-Induced Mitochondrial Dysfunction in Embryonic Hippocampal Development

Isotretinoin (13-cis-retinoic acid) is a synthetic retinoid widely prescribed for severe acne but it is also a potent teratogen. Its neurotoxic potential during pregnancy is strongly linked to mitochondrial dysfunction, particularly in the developing hippocampus-a region essential for memory formation, synaptic plasticity, and emotional regulation.

The mechanisms of isotretinoin-induced mitochondrial dysfunction include:

1. Excessive retinoic acid signaling: isotretinoin alters the balance of retinoic acid signaling, disrupting transcriptional regulation of genes required for mitochondrial biogenesis and function. Overactivation of retinoid receptors (RARs and RXRs) modifies expression of mitochondrial enzymes critical for oxidative phosphorylation (Crandall *et al.,* 2004).
2. Oxidative stress and ROS accumulation: isotretinoin increases mitochondrial production of reactive oxygen species (ROS), overwhelming embryonic antioxidant defenses. The hippocampus with its high metabolic demand, is particularly vulnerable to oxidative damage leading to lipid peroxidation and DNA injury (Ishikawa *et al*., 2008).
3. Impaired ATP production: inhibition of mitochondrial respiratory chain complexes decreases ATP availability. This impairs neuronal proliferation, dendritic spine formation, and synaptic connectivity, processes essential during embryonic hippocampal development.
4. Mitochondrial-mediated apoptosis: isotretinoin triggers release of cytochrome c and activation of caspase pathways, leading to premature apoptosis of hippocampal progenitor neurons (Collins and Mao, 1999). This contributes to reduced hippocampal volume and disrupted histoarchitecture.

5. Mitochondrial DNA (mtDNA) damage: increased oxidative stress from isotretinoin exposure induces mtDNA mutations and impairs replication, leading to defective mitochondrial inheritance during hippocampal neurogenesis.

2.5.1 Consequences of Mitochondrial Dysfunction for Hippocampal Development

~Structural changes: it leads to reduced neuronal density, impaired dendritic arborization, and altered laminar organization.

~Functional deficits: it causes impaired long-term potentiation (LTP) and hippocampal-dependent learning and memory.

~Behavioral outcomes: offspring prenatally exposed to isotretinoin show anxiety-like behavior, cognitive impairment, and learning disabilities consistent with hippocampal dysfunction (Lammer et al., 1985; Adams and Lammer, 2012).

In summary, isotretinoin exposure during embryogenesis disrupts hippocampal development by inducing mitochondrial dysfunction through oxidative stress, impaired mtDNA damage. These alterations compromise hippocampal structure and function, contributing to the long-term cognitive and behavioral deficits observed in prenatally exposed offspring. This highlights the role of mitochondria as critical mediators of isotretinoin-induced neurotoxicity and provides a mechanistic basis for the strict contraindication of Accutane use during pregnancy.

2.6. Neuronal Damage as a Consequence of Mitochondrial Dysfunction

Neuronal damage in embryonic development represents the downstream effect of mitochondrial impairment. The failure of mitochondrial homeostasis triggers several interlinked pathological processes:

1. Oxidative stress: ROS accumulation damages neuronal membranes, organelles, and genetic material. This accelerates neuronal loss in regions such as hippocampus and cortex, which are critical for memory and cognition (Kumar *et al*., 2019).
2. Excitotoxicity: ATP depletion compromises glutamate reuptake, prolonging excitatory signaling. Excessive activation of NMDA receptors allows uncontrolled calcium influx, activating proteases and causing neuronal degeneration (Olney, 2002).
3. Apoptotic pathways: the release of cytochrome c from damaged mitochondria activates caspase cascades, resulting in programmed neuronal death. Excessive apoptosis during development reduces the neuronal pool, altering circuit formation (Hansen *et a*l., 2010).

2.6.1 Consequences of Mitochondrial Dysfunction for Brain Development

The combination of mitochondrial dysfunction and neuronal damages produces long-term neurodevelopmental outcomes:

~ Impaired neurogenesis: fewer neurons available for cortical and hippocampal networks.

~Synaptic deficits: disrupted synaptogenesis and dendritic branching impair learning and memory.

~Neurodevelopmental disorders: it is linked to conditions such as autism, schizophrenia, and intellectual disability (Selemon and Zecevic, 2015).

In summary, mitochondrial dysfunction serves as an upstream trigger, while neuronal damage represents its downstream manifestation during embryonic development. Together, they disrupt the delicate processes of neurogenesis and circuit maturation, making the embryonic brain highly vulnerable to teratogens such as isotretinoin.

Understanding this cascade is essential for exploring antioxidant-based interventions, such as vitamin E and quercetin, which may mitigate prenatal neuronal injury.

2.7. Patterns of Hippocampal Injury

The hippocampus, located in the medial temporal lobe, plays a central role in learning, memory consolidation, and spatial navigation. Its unique architecture, consisting of the dentate gyrus (DG), cornu ammonis (CA1-CA3 subfields), and subiculum, makes it highly susceptible to injury during embryonic and postnatal development.

Hippocampal injury can occur due to hypoxia, excitotoxicity, oxidative stress, or exposure to teratogens such as isotretinoin. Distinct patterns of hippocampal vulnerability have been identified, reflecting regional differences in metabolic demand, receptor expression, and neuronal plasticity (Bartsch and Wulff, 2015).

2.7.1 Regional Vulnerability

1. CA1 REGION ("Sommer's Sector")

- The CA1 pyramidal neurons are the most sensitive to hypoxia-ischemia and excitotoxic injury.
- High density of glutamate (NMDA) receptors makes this region particularly prone to calcium-mediated neurotoxicity.
- Injury here leads to profound impairments in episodic memory and spatial orientation (Squire *et al*., 2004).

2. CA3 REGION

- CA3 neurons, which receive mossy fiber inputs from the dentate gyrus, are highly vulnerable to oxidative stress.
- Damage in this region disrupts pattern completion and associative memory.

3. DENTATE GYRUS (DG)

- The DG contains proliferating neural progenitors, making it a site of neurogenesis throughout life.

- It is particularly sensitive during embryonic development, where toxic insults impair neurogenesis, leading to long-term deficits in learning and memory flexibility (Jessberger and Parent, 2015).

4. SUBICULUM

- Though less studied, the subiculum serves as the main output pathway of the hippocampus.
- Injury here disrupts communication between the hippocampus and cortical/ limbic regions, amplifying cognitive dysfunction.

2.7.2 Cellular and Molecular Patterns of Injury

- Excito-toxicity injury: excessive glutamate signaling causes dendritic beading, spine loss, and neuronal swelling, especially in the CA1
- Oxidative damage: mitochondrial dysfunction in hippocampal neurons leads to lipid peroxidation and DNA damage, primarily affecting CA3 and DG.
- Apoptotic cell death: activation of caspase pathways causes selective neuronal loss, reducing hippocampal volume.
- Impaired neurogenesis: in the DG, toxic insults suppress proliferation of neural progenitor cells, altering hippocampal plasticity (Rai *et al.*, 2010; Wang *et al.*, 2014).

2.7.3 Functional Consequences of Hippocampal Injury

- CA1 damage: episodic memory deficits and disorientation.
- CA3 damage: impaired associative memory and stress susceptibility.
- Dentate Gyrus injury: reduced neurogenesis and poor adaptability to new learning tasks.
- Subiculum damage: disrupted cortical communication, contributing to widespread cognitive impairment.

In summary, patterns of hippocampal injury demonstrate that not all regions are equally vulnerable; the CA1 sector, CA3 neurons, and dentate gyrus are disproportionately affected by oxidative stress, excito-toxicity, and teratogenic exposure. Understanding these selective vulnerabilities is crucial in evaluating isotretinoin-induced prenatal hippocampal toxicity and exploring the neuroprotective roles of antioxidants such as Vitamin E and quercetin (Squire *et al.,* 2004; Eichenbaum, 2017).

2.8. Neurobehavioral Tests in the Assesment of Hippocampal Injury

Neuro-behavioral (neuropsychological) testing being an accepted methodology for evaluating the functional integrity of the central nervous system have been widely used to evaluate subclinical neurotoxic effects on cognition, memory, alertness, executive function, mood, psychomotor abilities (Anger and Cassitto,1993; Mergler *et al*.,1996; WHO, 2001). Early in the 1960s, neurobehavioral testing which was based on experimental and clinical psychology and neuropsychology, was developed to detect developing impairment and prevent further deterioration (Iregen, 1994). There is an extensive spectrum of neuropsychological testing available and the choice needs to be modified for each case (Anger and Cassitto, 1993).

Connections between different neural system regions shape behavior. A modification to interrogational communicational, the central nervous system, or any physiological pathology might result in behavioral activities that will be performed (Fornito *et al.,* 2015). Some of the neuro-behavioral tests conducted in this study are:

- **Elevated Plus Maze Test (EPM):** This is an assessment tool designed to evaluate unconditioned responses of anxiety in laboratory animals (Staimer, 2011). It usually uses rodents as a screening test for potential anxiolytic or anxiogenic substance and as a general study tool in neuro-behavioral anxiety research such as post-traumatic stress disorders (PTSD) and traumatic brain injury (TBI) (Ojo and Mouzon, 2016). The EPM is a model of

behavior that doesn't need animal training and has a high validity based on ethology (Staimer, 2011). This test was an initial one created by Handley and Mithani and is often utilized when evaluating anxiety (Handley and Mithani, 1984). After the researchers acknowledged the test's development in 1986 and revealed the test did in fact have the ability to measure anxiety. The measurement methodology still makes active use of the EPM test of anxiety (Pellow and File, 1986). The hippocampus, limbic regions, amygdala, dorsal raphe nucleus and other brain sites as well as the mechanisms (such as glutamate, GABA, serotonin, and neuromodulators of the hypothalamic-pituitary-adrenal axis, underlying anxiety behavior can be studied using the elevated plus maze as a behavioral assay (Pellow *et al*., 1985; Handley and Mithani, 1984; Silva and Brandao, 2000). The test apparatus is elevated above the floor with two open and closed arms. This technique is developed to measure the evasion of exposed, bright and elevated areas by experimental animals. In addition, using this test, the animals' openness to explore new environment can also be evaluated (Fornito *et al*., 2015). The conduct of avoiding open environment during the EPM tests suggest avoiding situations that cause physiological stress, particularly if the animal is restricted to this environment, corticosterone levels and defecations increase in relation to anxiety (Fornito *et al*., 2015). The amount of time the animals spend in the open and closed arms during the test indicates their level of anxiety, while the quantity of openings in the arms and the animals' impromptu motor movements are measured. Furthermore, ethological standards like the amount dropping stools, head dipping and frozen or tight positions can also be assessed with this test (Walf and Frye, 2007).

- **Y maze Test:** The spontaneous alternation test is a popular tool for researching motivation and memory. This test makes use of the natural tendency to switch between signals and objects in the absence of explicit reward or penalties (Lalonde, 2002; Hughes, 2004). The Y-maze test is one of the several methods for conducting the spontaneous alternation test that is

frequently utilized because it doesn't require training and is less likely to produce anxiety or tension (Hughes, 2004; Heredia-Lopez *et al.*, 2016). Mice can be tested for short-term memory using the Y-maze (Kraeuter *et al*., 2019). Test mice can also be used to access spatial reference memory, which is underlined by the hippocampus. The arms of the Y-maze are spaced 120 degrees apart to allow rodents to freely explore (Lalonde, 2002; Hughes, 2004). By having one arm closed during training in the Y-maze. Following a one-hour intertrial break, the mouse ought to remember which arm it has previously not visited and explore the arm more often (Kraeuter *et al*., 2019). This leads to repeated trips to all three arms that was not previously visited. It has been suggested that a high proportion of alternation is a sign of strong spatial memory (Jordan *et al*., 2022; Dillion *et al*., 2008; Prieur and Jadavji, 2019). In the past, visual scoring has been used to analyze spontaneous alternation behavior on the Y-maze. This involves counting the number of arm entries and calculating the rate of selecting the arm that hasn't been visited as much in order to compare locomotive activity between the control and experimental groups (Jordan *et al* 2022; Heredia-Lopez *et al.*, 2016; Prieur and Jadavji, 2019). An intact memory mouse which also has an intact prefrontal cortex functioning will recall the arms it has previously visited and will exhibit a propensity to enter a less visited arm (Kraeuter *et al.*, 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Animals

In this study, 20 females adult juvenile Wistar rats with an average body weight of 155g were used. The rats were bred in the animal house of the department of Anatomy, school of Basic Medical Sciences, University of Benin, Benin City. There, they were housed in plastic cages under standard condition with 12 hours light and 12 hours dark cycle and given water and Grower's marsh every day, which was produced by premier feed mills co LTD, a division of flour mills of Nigeria Plc and water *ad libitum*. They were allowed to acclimatize for two weeks before the commencement of the experiment.

The animals were mated with male juvenile Wistar rats on the evening of their pro-estrous phase of the estrous cycle (~9-15 hours).

After mating, pregnancy was confirmed by vaginal smear and subsequent weight gain. The confirmed pregnant rats were grouped based on their weights.

3.2 Experimental Materials

Plastic cages, ceramic plates, syringe, weighing scale, chloroform, surgical gloves, surgical scissors, cotton wool, beakers, pipette, universal bottles, plain bottles, laboratory cages, sawdust, formalin, slides, paraffin wax, normal saline, oral gavage, dissection table, refrigerator, rotary microtome, microscope.

3.3 Experimental Design and Procedure

The twenty (20) Wistar rats were divided into four (4) groups, three experimental groups and one control group. All four groups were labelled A-D. The control group was tagged group A while the experimental groups were tagged B-D.

Group A (control group) animals were the control group.

Group B animals were treated with 10mg/kg body weight of Accutane.

Group C animals were offspring of dams treated with 10mg/kg body weight of Accutane plus 500mg/kg body weight of Quercetin plus 500mg/kg body weight of Vitamin E.

Group D animals were offspring of dams treated with 10mg/kg body weight of Accutane plus 500mg/kg body weight of Vitamin E.

Administration of the drug was with the aid of an orogastric tube for only 7 days from gestation day 14-21.

3.4 Sourcing and Collection of Drugs

Accutane/Isotretinoin (13-cis retinoic acid), Quercetin, and Vitamin E (alpha-tocopherol) were obtained from a registered pharmacy in Awka, Anambra state, Nigeria and transported to Benin City, Edo State.

3.5 Drug Preparation and Administration

The drug (Accutane) and the antioxidants (Quercetin and Vitamin E) were dissolved in water at a standardized drug-to-solvent ratio calculated according to their respective milligram concentrations.

Administration of drug was done using an oral gavage to ensure precise treatment delivery. The dosage was 10mg/kg body weight of Accutane, 50mg/kg body weight of quercetin and 500mg/kg body weight of vitamin E.

3.6 Neuro-Behavioural Tests

The neuro-behavioral tests (Y-maze, Novel Object Recognition and Elevated Plus Maze Tests) were carried out.

3.6.1 Y-Maze Test

Fig. 3.1: Y-Maze (Ann-Katrin *et al*., 2019).

The Y-maze can be used to test for short-term memory in mice. Allowing mice to explore all three arms of the maze allows for the assessment of spontaneous alternation, a measure of spontaneous alternation, a measure of spatial working memory that is prompted by rodent's natural eagerness to explore previously unvisited areas (Ann-Katrin *et al.*,2019). The three arms of the Y-maze are spaced 120 degrees apart to allow rodents to freely explore. The methodology involves placing the animal at the start of arm B allowing it to navigate the Y-maze for 5 or 8 minutes respectively while being monitored by an automated tracking system. (Hvoslef-Eide *et al.*, 2016). Each trial begins promptly and ends after a specific time. Scoring entails documenting every arm entry, defined as all four paws entering an arm. After the trial, the animal is returned back to the cage and the number of fecal pellets is noted. The testing arena is sanitized before each subsequent trial (Hvoslef-Eide *et al.*, 2016).

3.6.2 Elevated plus maze test (EPM)

Fig. 3.2: EPM Test (Munekazu *et al.,* 2008)

The elevated plus maze test is one of the most popular tests for evaluating anxiety like behavior. The EPM is verified for mice (Lister, 1987) and rats (Pellow *et al*., 1985). The test is based on the mice's innate dislike of wide spaces and elevations, 38 as well as on their impulsive natural curiosity in unfamiliar environments (Munekazu *et al.,* 2008). This is a five-minute economic test. Apparatus: The EPM was constructed from dark grey PVC and had two opposed open arms measuring 50cm by 12cm and two opposite closed arms encircled by walls that were 50cm high and had the same measurements. The center portion, which enables the animal to move from arm to arm, was a square measuring 12cm by 12cm. To guarantee that no animal would fall from the maze, 0.5 x 0.5 cm ledges were installed in the open arms of the elevated maze, which was 50cm above the ground (Schneider *et al*., 2011). It was possible to move the equipment across the room and every trial was conducted with the same positioning and illumination. The trials were captured on film and computer analysis was done to track the amount of time spent in and visits to the EPM arms. An expect observer carried out the remaining ethological analyses. The computer equipment was located outside the experiment rooms. Every experiment was conducted between 1100 and 1600 hours, in the midst of the light phase (Schneider *et al.,* 2011).

3.7 Termination of Experiment

On postnatal day thirty-one (PND 31), the animals were humanely sacrificed under light anesthesia. The weight of each animal was taken and recorded as their final weight. The brains of these animals were harvested and weighed, then placed inside tissues bottles with appropriate labelling for easy identification. The brains were fixed inside the bottles containing formal saline. The brains were also collected for oxidative stress analysis.

3.8 Histological Procedures

3.8.1 Paraffin tissue processing of Drury and Wallington (1980).

Following the fixation of the harvested tissues (brain) in 10% formalin, the tissues were allowed to stand for about 72hours to achieve good tissue penetration, the tissues were processed as follows;

- Dehydration of tissues in an increasing grade of alcohol (from 70% to 90% and absolute alcohol) using ethanol as the choice of alcohol.
- Xylene as a clearing agent was used in clearing alcohol. Tissues were allowed to pass through two changes for the removal of alcohol from tissue samples.
- The tissues were infiltrated in three changes in a solution of molten paraffin wax in an oven at a temperature of 65-70℃. The first two changes were carried out for 15 mins each while the last change was for about 30 minutes.
- Embedding was carried out using an embedding mould, into which the molten paraffin wax was poured and the infiltrated tissues were placed in it in a longitudinal orientation to produce longitudinal section.
- The molten paraffin wax was allowed to cool, which then result in solidification to form tissue blocks.
- Sectioning of the tissue blocks was done with the aid of a rotary microtome to cut tissue into thin-like sections of 5 microns' thickness.

3.8.2 Hematoxylin and eosin staining method of Drury and Wallington (1980).

- Satisfactory and good tissue sections which came out as ribbon were selected and placed in 20% alcohol for spreading of the paraffin sections which are then cut and floated in a water bath at a temperature of 30℃

. • The sectioned tissues were taken up on glass slides and allowed to air-dry.

- The tissue sections were placed in xylene for 15 minutes to remove excess paraffin wax from the tissues and were subjected to hydration by passing them through descending grades of alcohol (100%, 90% and 70%) and then into water, all of which lasted for about 5 minutes each.
- Staining of the tissue was done using H&E dyes. The tissues were stained in hematoxylin for 10 minutes.
- Tissues were washed in running tap water (a process called bluing).
- Sections were counter-stained with 1% Eosin for 5-10 minutes.
- Tissues were then rinsed in water.
- The tissues were followed up by rapid dehydration through ascending grades of alcohol (from 70% to 90% and absolute alcohol) for 5 minutes.
- Tissues were finally cleared using xylene for 5 minutes and the slides were mounted with glass cover slip using a suitable mountant, Distrene Plasticizer and Xylene (DPX).

3.9 Data Analysis

The data analysis was done using the IBM statistical package for social science (SPSS) version 22. The animal body weights were analyzed, the relative brain weights were analyzed using the one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison tests. Tables and graphs were used for result presentation, and values were considered significant at $p<0.05$. All the data generated were used for the analysis and each animal study group was considered as a single experimental unit.

CHAPTER FOUR

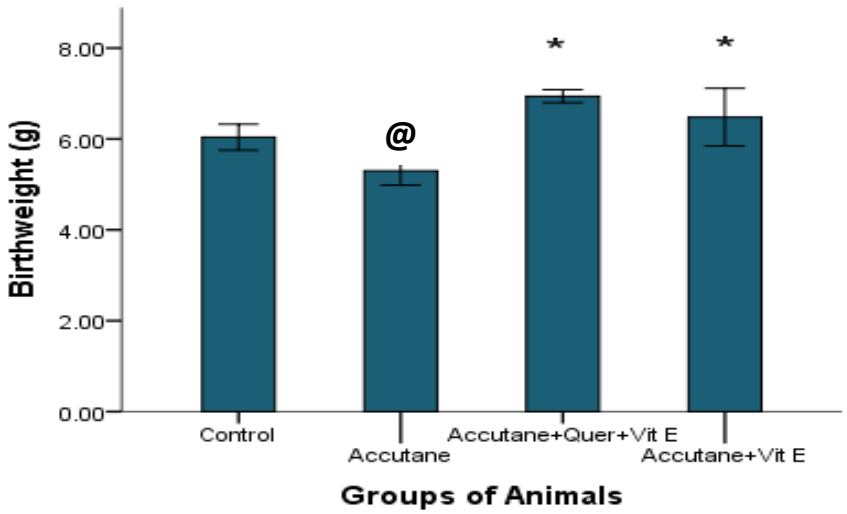
RESULTS

4.1 Birth Weight

Results obtained showed that Accutane caused significant decrease ($p<0.05$) in the birth weight of animals treated with 10mg/kg body weight of Accutane only (group B) when compared to control (group A). However, group C (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) and group D (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) showed a significant increase compared to group B (10mg/kg body weight of Accutane only).

4.2 Body Measurements

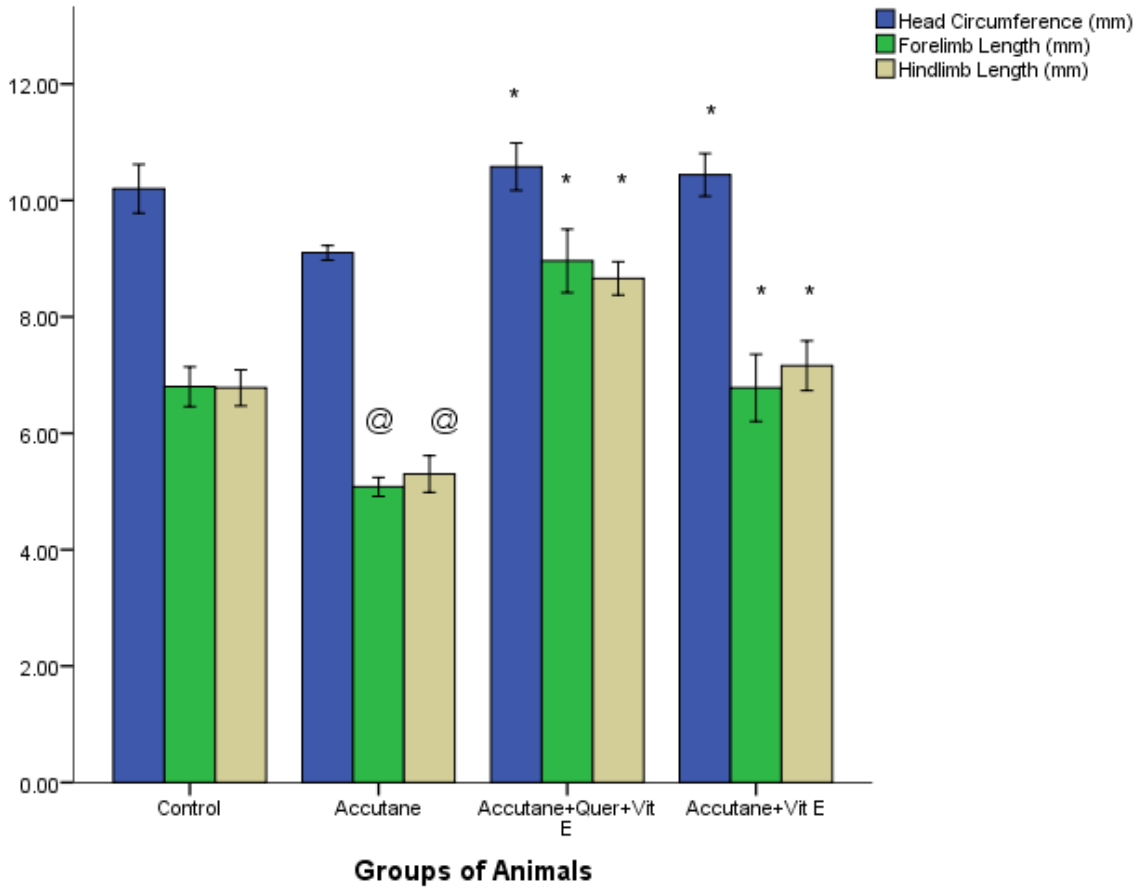
Results obtained showed that the head circumference, forelimb length and hindlimb length of group B (10mg/kg body weight of Accutane) were decreased ($p<0.05$) when compared to group A (control). However, group C (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) and group D (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) were significantly increased compared to group B (10mg/kg body weight of Accutane).



Bar Chart 4.1 showing birthweight of fetuses

@ indicates $p<0.05$ compared to control

*indicates $p<0.05$ compared to Accutane only



Bar Chart 4.2 showing head circumference, forelimb and hindlimb length

@ indicates $p<0.05$ compared to control

*indicates $p<0.05$ compared to Accutane only

4.3 Neurobehavioral Tests

4.3.1 Transfer Latency

Results obtained shows that there is a significant increase in transfer latency ($p<0.05$) in group B (10mg/kg body weight of Accutane) when compared to group A (control). However, there was only a slight difference in group C animals (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) and group D animals (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) when compared to group B animals (10mg/kg body weight of Accutane).

4.3.2 Spontaneous Alteration

Results obtained shows that there is a significant decrease in spontaneous alteration ($p<0.05$) in group B (10mg/kg body weight of Accutane) when compared to group A (control). However, there was also a significant increase in group C animals (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) and group D animals (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) when compared to group B animals (10mg/kg body weight of Accutane).

4.3.3 Open Arm Entries

Results obtained shows that there is a significant decrease ($p<0.05$) in treated group B animals (10mg/kg body weight of Accutane) when compared to group A animals (control), group C animals (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) and group D animals (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E).

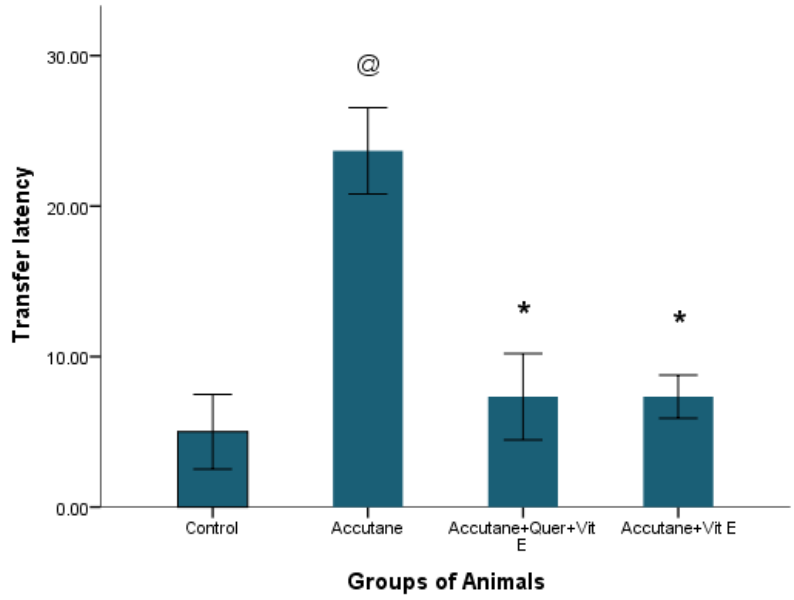
4.3.4 Closed Arm Entries

Results obtained shows that there is an increase ($p<0.05$) in group B animals (10mg/kg body weight of Accutane) when compared to group A animals (control) and group C animals (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body

weight of Vitamin E). however, there was no significant difference ($p<0.05$) in group D animals (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) when compared to group B animals (10mg/kg body weight of Accutane).

4.3.5 Open/Closed Arm Quotient

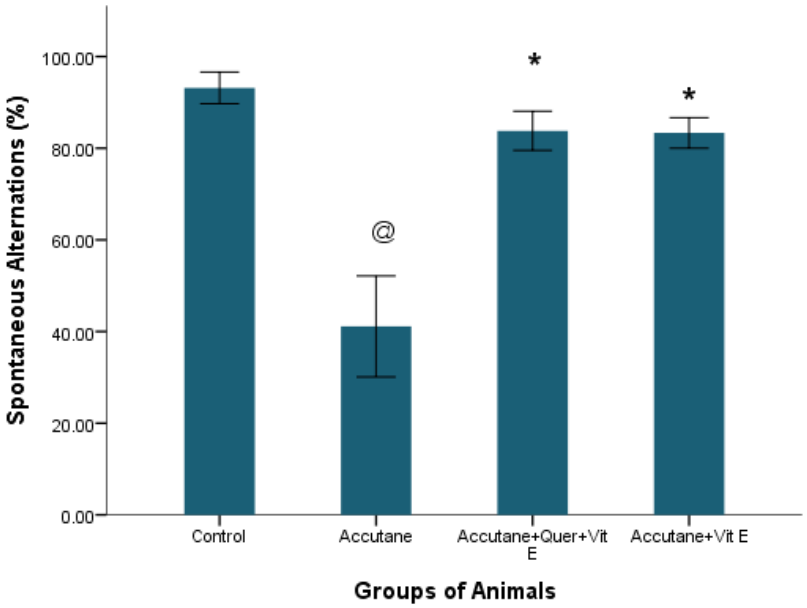
Results obtained shows that there is a significant decrease ($p<0.05$) in group B animals (10mg/kg body weight of Accutane) when compared to group A animals (control). However, there was no difference in group C animals (10mg/kg body weight of Accutane+50mg/kg body weight of Quercetin+500mg/kg body weight of Vitamin E) when compared to group A animals (control) and only a slight difference in group D animals (10mg/kg body weight of Accutane and 500mg/kg body weight of Vitamin E) when compared to group A animals (10mg/kg body weight of Accutane).



Bar Chart 4.3 showing transfer latency

@ indicates $p<0.05$ compared to control

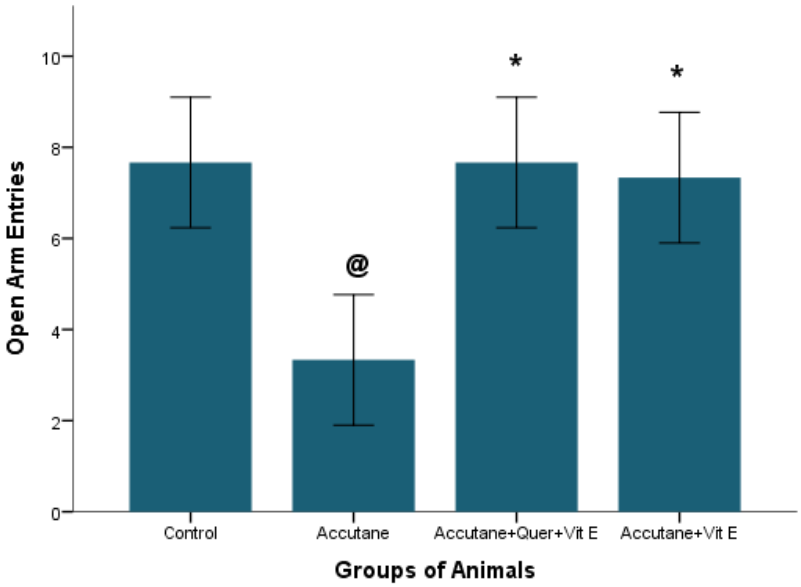
*indicates $p<0.05$ compared to Accutane only



Bar Chart 4.4 showing spontaneous alteration

@ indicates $p<0.05$ compared to control

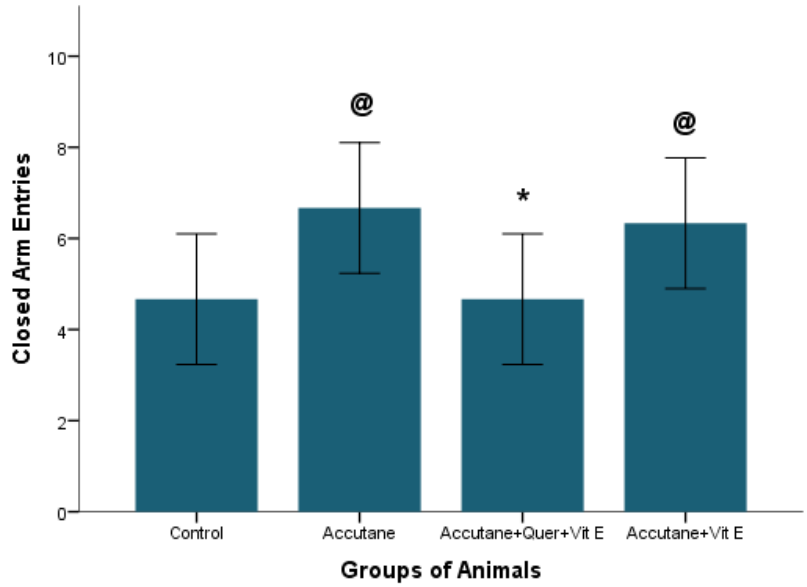
*indicates $p<0.05$ compared to Accutane only



Bar Chart 4.5 showing open arm entries

@ indicates $p<0.05$ compared to control

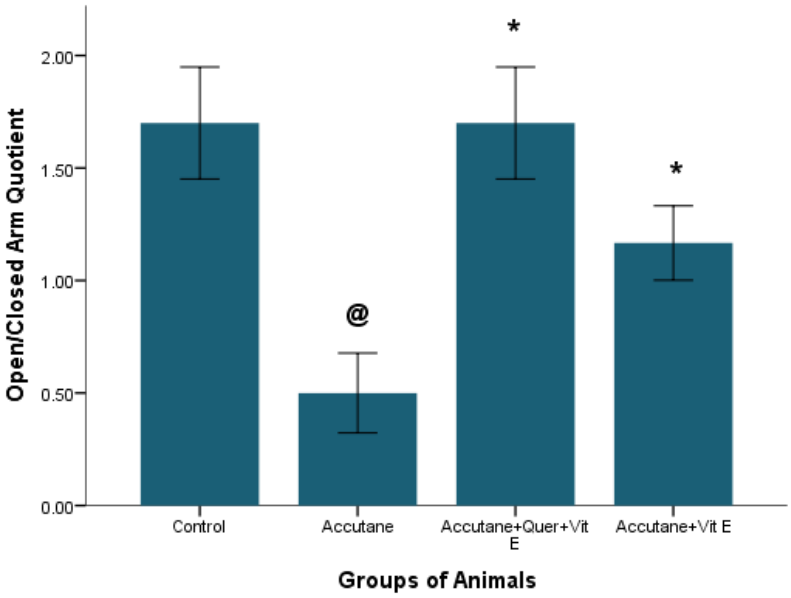
*indicates $p<0.05$ compared to Accutane only



Bar Chart 4.6 showing closed arm entries

@ indicates $p<0.05$ compared to control

*indicates $p<0.05$ compared to Accutane only



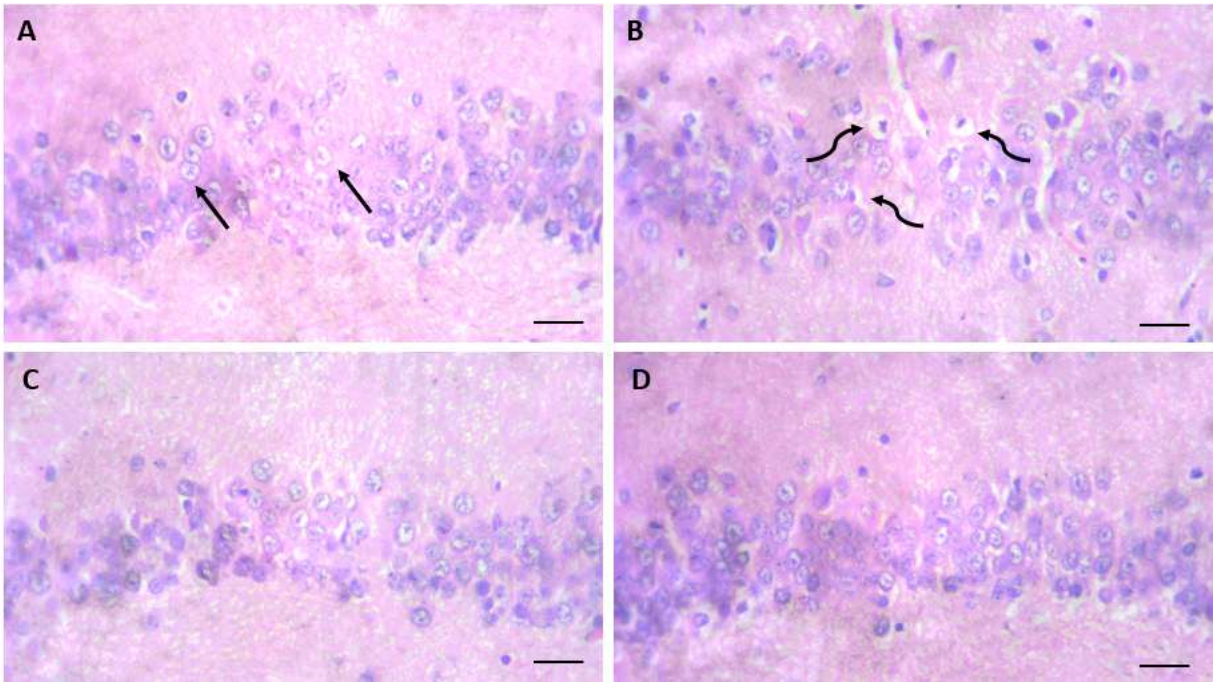
Bar Chart 4.7 showing open/closed arm quotient

@ indicates $p<0.05$ compared to control

*indicates $p<0.05$ compared to Accutane only

4.4 Histology

Plate 4.1: Representative histology of the hippocampus CA1 in control and treatment rats. (A) Control group revealing normal structure of pyramidal cells (arrows). (B) Accutane-treated rats showing atrophy and vacuolated pyramidal cells and astrocytes (curved arrows). (C-D) Relatively normal histological structure of the hippocampus observed. (H&E; Scale bar: 25µm)



CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

In this study, the neuroprotective effects of Vitamin E and Quercetin against Accutane (isotretinoin)-induced prenatal hippocampal toxicity were evaluated using behavioral, biochemical, and histological assessments in Wistar rats. The results revealed that prenatal exposure to isotretinoin led to significant neurobehavioral and histopathological alterations, while co-administration with Vitamin E and Quercetin attenuated these adverse effects.

The parameters assessed included birth weight, head circumference, hind limb and forelimb length and neurobehavioral indices such as open and closed arm entries, open/closed arm quotient, spontaneous alterations and transfer latency.

A significant reduction in birth weight was observed in the Accutane-treated group compared to the control group. This finding is consistent with previous studies that linked isotretinoin exposure to impaired fetal growth and developmental delay due to retinoid-induced oxidative stress and mitochondrial dysfunction (Ali *et al*., 2020). Conversely, co-treatment with Quercetin and Vitamin E resulted in an improvement in birth weight, suggesting that their antioxidant and anti-inflammatory action mitigated the teratogenic effects of Accutane (Zhao *et al.*, 2021).

Similarly, head circumference showed a noticeable reduction in the Accutane-treated group, indicating potential neurodevelopmental delay and altered brain growth. These effects may result from isotretinoin's interference with retinoic acid signaling pathways that regulate neuronal differentiation and proliferation (Halevy *et al*., 2019). The groups co-administered with Quercetin and Vitamin E, however, exhibited significant restoration in head circumference values, suggesting enhanced neuroprotection and preservation of neuronal integrity (Park *et al*., 2020).

The hind limb and forelimb lengths of neonates in the Accutane-only group were significantly reduced, reflecting general growth retardation and possible musculoskeletal developmental toxicity. These results agree with earlier reports that isotretinoin exposure disrupts limb morphogenesis during embryogenesis (Chung *et al.,* 2018). The improved limb measurements in the Quercetin +Vitamin E group can be attributed to their ability to neutralize free radicals and restore mitochondrial function, both of which are critical for energy-dependent tissue growth during gestation (Fahmy *et al*., 2021).

Behavioral assessment through the elevated plus maze revealed a significant decrease in open arm entries and an increase in closed arm entries in the Accutane-treated rats, indicating elevated anxiety levels and hippocampal dysfunction. The pattern aligns with studies showing that hippocampal injury leads to reduced exploratory behavior and increased anxiety (Rogawski and Wenk, 2020). However, rats treated with Quercetin and Vitamin E showed an increased number of open arm entries and a higher open/closed arm quotient, suggesting the neuroprotective effects consistent with improved hippocampal function (Zhang *et al.,* 2022).

The spontaneous alteration test, a measure of spatial working memory, showed a significant decline in the Accutane group, indicating impaired hippocampal-dependent memory processes. This is supported by earlier findings that isotretinoin-induced neuronal apoptosis can impair synaptic plasticity (Wu *et al.,* 2021). Quercetin and Vitamin E co-administration significantly increased the percentage of spontaneous alternations, demonstrating improved memory and learning, likely through their antioxidative and membrane-stabilizing effects (Lee *et al.,* 2019).

Furthermore, transfer latency in the elevated plus maze was significantly prolonged in the Accutane group, reflecting poor learning and memory consolidation. This agrees with studies showing that isotretinoin exposure leads to deficits in hippocampal long-term potentiation (LTP) (Nelson *et al.,* 2020). The shortened transfer latency observed in the Quercetin and

Vitamin E-treated groups indicates enhanced cognitive performance and hippocampal restoration.

The observed neurotoxicity in the isotretinoin-treated group may be attributed to the drug's ability to generate excessive reactive oxygen species (ROS) and lipid peroxidation, leading to mitochondrial dysfunction in neuronal tissues. This agrees with the findings of Sato *et al.* (2020), who reported that isotretinoin disrupts neuronal homeostasis through increased ROS production and apoptosis. Similar observations were made by O'Reilly *et al*. (2018), who linked isotretinoin exposure to neurodevelopmental abnormalities in experimental models.

Histological examination of the hippocampus revealed neuronal degeneration, cellular shrinkage, and disorganization of pyramidal cell layers in the Accutane-only group, while the groups treated with Vitamin E and Quercetin showed preserved cytoarchitecture and normal cellular arrangement. These findings are consistent with the report of Mahmoud *et al.* (2022), who restored normal morphology following exposure to teratogenic agents.

5.2 Conclusion

Findings from the present study demonstrate that prenatal Accutane exposure induces dose-dependent neurodevelopmental deficits and behavioral abnormalities, primarily through mitochondrial dysfunction, and neuronal apoptosis. The co-administration of Quercetin and Vitamin E provided significant neuroprotection, as shown by improvements in growth indices, neurobehavioral performance, and hippocampal function. This proves that both compounds serve as effective agents against Accutane-induced prenatal hippocampal neurotoxicity.

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