BEHAVIOURAL AND ANXIOTROPIC EFFECTS OF ORPHENADRINE AND DICLOFENAC IN ACUTE PAIN IN SWISS ALBINO MICE

BY

NWACHUKWU ONYEDIKACHI ONYEUKWU

PHA1908544

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

FACULTY OF PHARMACY

UNIVERSITY OF BENIN

BENIN CITY

SEPTEMBER, 2025.

BEHAVIOURAL AND ANXIOTROPIC EFFECT OF ORPHENADRINE AND DICLOFENAC IN ACUTE PAIN IN SWISS ALBINO MICE

BY

NWACHUKWU ONYEDIKACHI ONYEUKWU

PHA1908544

A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, FACULTY OF PHARMACY, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHARMACY (PHARM.D) DEGREE.

SEPTEMBER, 2025.

CERTIFICATION

We the undersigned hereby certify that this work was carried out by **NWACHUKWU ONYEDIKACHI ONYEUKWU** with matriculation number **PHA1908544**, in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City.

---------- ----------

Dr. Osaze Edosuyi **Date**

(Project Supervisor)

---------- ----------

Dr. Adaeze P. Uchendu **Date**

(Head of Department)

ANTI-PLAGIARISM CERTIFICATION

We the undersigned attest and declare that the thesis of **NWACHUKWU ONYEDIKACHI ONYEUKWU** titled **BEHAVIOURAL AND ANXIOTROPIC EFFECTS OF ORPHENADRINE AND DICLOFENAC IN ACUTE PAIN IN SWISS ALBINO MICE** has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

---------- ----------

Nwachukwu Onyedikachi Onyeukwu **Date**

(Student)

---------- ----------

Dr. Osaze Edosuyi **Date**

(Project Supervisor)

---------- ----------

Dr. Adaeze P. Uchendu **Date**

(Head of Department)

DEDICATION

I dedicate this work first to Almighty God for His wisdom, guidance and inspiration provided throughout my academic journey and the course of this work.

To my ever-supportive family, whose steadfast encouragement, prayers, and resources have provided the platform on which I stand today

ACKNOWLEDGEMENTS

I wish to express my most sincere gratitude to my project supervisor, Dr. Osaze Edosuyi, for his patience, exceptional guidance, and valuable input, throughout this research. Your tutelage sir, went a long way in securing the success and completion of this work.

My thanks also go to the lecturers and staff of the Faculty of Pharmacy, University of Benin, for their dedication to teaching and the academic support they have provided during these years of our studies.

A heartfelt thank you to my family for their constant presence—through financial assistance, prayers, regular check-ins, and unwavering moral and emotional support. God bless you all immensely.

To my brother in arms, Izogie Bright: I deeply appreciate your motivation and practical help during the most difficult moments. Your support will always mean a great deal to me

I would also like to extend my sincere appreciation to my project partners—singles and couples alike—for their cooperation and shared efforts in bringing this research to completion.

Above all, I give thanks to God Almighty for His grace, love, and comfort throughout this journey.

All praise be returned to him in the end

TABLE OF CONTENTS

TITLE PAGE……. i

CERTIFICATION……. iii

ANTI-PLAGIARISM CERTIFICATION……. iv

DEDICATION……. v

ACKNOWLEDGEMENTS……vi

TABLE OF CONTENTS…….. vii

LIST OF FIGURES……ix

LIST OF TABLES……. x

ABSTRACT……... xi

CHAPTER ONE..... 1

1.0.INTRODUCTION……1

1.1. Pain and Behaviour……..…….....1

1.2. Biological Basis for Interaction between Pain, Behaviour and Anxiety……...……... 1

1.2.1. The Sensory-Discriminative Pathway……...…….... 2

1.2.2. The Affective-Motivational Pathway……...……..... 3

1.2.3. Biochemistry of Nociception.....……...…….....6

1.3. Drugs Under Study: Chemistry and Pharmacology……..……... 8

1.3.1. Orphenadrine…….…… 8

1.3.1.1. Pharmacodynamics…… 8

1.3.1.2. Pharmacokinetics…….…….…….9

1.3.1.3. Indications.....…….…… 9

1.3.1.4. Safety Profile…….…… 10

1.3.1.5. Contraindications……...……..10

1.3.2. Diclofenac ……..…….11

1.3.1.1. Pharmacodynamics ……...……..11

1.3.1.2. Pharmacokinetics……..12
1.3.1.3. Indications……13
1.3.1.4. Safety Profile……13
1.3.1.5. Contraindications……..13
1.4. Preclinical Models of Acute Pain in Rodents……14
1.5. Behavioural Assays of Pain in Rodents……16
1.6. Biochemical Correlates: Oxidative Stress…….. 18
1.7. Malondialdehyde as a Biomarker of Oxidative Stress in Acute pain and Anxiety…… 20
1.8. Justification of the Study…… 22
1.9. Aim and Objectives…… 23
CHAPTER TWO……... 24
2.0. MATERIALS AND METHODS……24
2.1. Materials …… 24
2.1.1. Drugs and chemicals …….. 24
2.1.2. Apparatus.....……...24
2.1.3. Experimental Animals…….. 24
2.2. Methods …… 24
2.2.1. Body Weight.....25
2.2.2. Grouping and Drug Administration.....25
2.2.3. Acetic Acid Induced Mouse Writhing Test.....25
2.2.4. Elevated Plus Maze (EPM) Test 26
2.2.5. Sample Collection..... 26
2.2.6. Biochemical Assay..... 26
2.2.7. Data Analysis..... 27
CHAPTER THREE..... 28
3.0. RESULTS..... 28
3.1. Effect Of Drug Treatments On Writhing Response..... 29
3.2. Effect Of Drug Treatments On MDA Levels 29
3.3. Effect Of Drug Treatments On Time Spent In Arms Of EPM 30
3.4. Effect Of Drug Treatments On Number Of Entries Into Arms Of EPM 31
CHAPTER FOUR..... 32

4.0.DISCUSSION……..…..... 32
CHAPTER FIVE……...…….....……..... 37

5.0. CONCLUSION……..…….……37
5.1. Contributions to knowledge……..……..38
5.2. Recommendations……..……38
REFERENCES…….…… 40

APPENDIX……..……..46

LIST OF FIGURES

Figure 1.1: Anatomical Overview of Pain Pathways..... 5

Figure 1.2: Chemical Structure of Orphenadrine..... 8

Figure 1.3: Chemical Structure of Diclofenac 11

Figure 1.4: Chemical Structure of Malondialdehyde 20

Figure 3.1: Writhing response over thirty (30) minutes across all treatment groups..... 28

Figure 3.2: Malondialdehyde levels across treatment groups in mmol/mg protein.....29

Figure 3.3: Time in seconds spent in the open and closed arms of the EPM..... 30

Figure 3.4: Number of entries into the open and closed arms of the EPM.....31

LIST OF TABLES

Table 2.1: Body Weight and Volume of Normal Saline (10ml/kg) administered to the first treatment group (control group, marked green)..... 46

Table 2.2: Body Weight and Volume of Orphenadrine (25mg/kg) administered to the second treatment group (marked blue)..... 46

Table 2.3: Body Weight and Volume of Diclofenac (50mg/kg) administered to the third treatment group (marked red)..... 47

Table 2.4: Body Weight and Volume of Orphenadrine (25mg/kg) and Diclofenac (50mg/kg) administered to the fourth treatment group (marked black)..... 47

ABSTRACT

Background: Acute pain is a complex experience involving both sensory perception and significant affective components, particularly anxiety. This interaction is mediated by shared neural pathways and potentially linked to oxidative stress. This study investigates the comparative anxiotropic effects of orphenadrine and diclofenac, alone and in combination, focusing on their ability to modulate anxiety-like behaviour and associated oxidative damage in acute pain in mice.

Methods: Twenty-four mice were allocated into four groups receiving normal saline (10ml/kg), orphenadrine (25mg/kg), diclofenac (50mg/kg), and an orphenadrine–diclofenac combination at the aforementioned doses. Peripheral analgesia was assessed with the acetic-acid–induced writhing assay while anxiety-like behavior was evaluated the following day, using the elevated plus maze. Animals were euthanized, brain tissue was excised and fixed in 10% formalin, and brain MDA was quantified by TBA derivatization and HPLC.

Results: Diclofenac and the orphenadrine-diclofenac combination demonstrated superior analgesic activity ($p < 0.01$ to $p < 0.001$). Both orphenadrine ($p < 0.0001$) and diclofenac ($p < 0.001$) were associated with significantly elevated MDA levels compared to the control group, while the combination group showed a reduction in MDA compared to either drug alone. All three treatment groups saw a decrease in the time spent in the open arm and a modest increase in time spent in the closed arms of the EPM, compared to the control group ($p < 0.001$). All drugs were associated with a decrease in the number of entries into the open arm, while orphenadrine uniquely led a significant reduction in closed arm entries ($p < 0.01$) compared to the control, an effect not seen with diclofenac or the combination groups.

Conclusion: Data from this study shows that all three analgesic treatments were associated with significantly increased anxiety-like behavior and accompanying oxidative damage.

Keywords: Orphenadrine, diclofenac, acute pain, behaviour, anxiotropic, oxidative stress, malondialdehyde.

CHAPTER ONE

1.0. INTRODUCTION

1.1. PAIN AND BEHAVIOUR

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). This definition, consistent with current concepts in the field of acute pain management, makes it clear that the experience of pain is more than a simple sensory phenomenon, but is rather more intricate, involving both physiological and psychological aspects (McQuay, 2009).

Pain is classified by a number of criteria including cause, function, anatomical location, duration of persistence etc. Based on its duration, pain can be categorised into transient, acute and chronic pain. Transient pain occurs when tissues respond to nociceptive stimuli, without any actual damage. Acute pain is characterized by a brief duration and results from tissue injury, while chronic or persistent pain is often caused by tissue injury or nerve damage that can surpass the body's ability to heal. (Xiao, Ding & Zhang, 2021).

From the physiological standpoint, the experience of pain is commonly known to be mediated through nociceptors, specialized nerve endings, which detect noxious stimulus and convert this perception into an electrical signal. This signal travels along a sensory nerve to the spinal cord, where it is passed to a second-order neuron which crosses to the opposite side of the spinal cord and ascend to the brain, primarily via the spinothalamic tract. In the brain, this signal is transmitted through the thalamus to the cortex, where it is processed for conscious perception of pain. (McQuay, 2009).

Yet, another important often overlooked aspect of the total pain experience exists, which is its affective or emotional, and consequently behavioural component, which is emphasised under the psychosocial model of pain, which posits that acute pain exists under larger psychosocial context that impacts how it

is experienced and its perceived intensity (McQuay, 2009). The interaction between pain and affective state is more common in chronic pain, as many patients with chronic pain often present with psychiatric comorbidities, particularly anxiety and depression. (Michaelides & Zis, 2019; Parent et al, 2012). However, this study is aimed at outlining the connection between behavior and pain, especially anxiety, in the acute pain setting, and test for possible pharmacological interventions, as the complex nature of pain requires the use of robust preclinical behavioral models for the development of new analgesic and anxiolytic medications (Larson et al., 2019; Parent et al, 2012).

1.2. BIOLOGICAL BASIS FOR INTERACTION BETWEEN PAIN, BEHAVIOUR AND ANXIETY

The processing of pain, known as nociception, has been known to engage a dedicated pathway from the periphery to the central nervous system (CNS) (McQuay, 2009), which consists of two major functional components: the sensory-discriminative, and the affective-motivational (Refsgaard et al., 2016), both of which are essential to the relationship between acute pain and affective behaviour, such as anxiety (Michaelides & Zis, 2019; Parent et al, 2012).

1.2.1. The Sensory-Discriminative Pathway

This pathway mediates the objective aspects of pain e.g. location, intensity, and duration etc.

1. **Peripheral Transduction**: Noxious stimuli (mechanical, thermal, chemical) are converted into electrical signals by nociceptors — specialised primary afferent neurons (McQuay, 2009).
2. **Spinal Cord Transmission:** These signals travel through A-delta (A**δ**) and C-fibres to the dorsal horn of the spinal cord (first-order neurons), where they synapse with second-order neurons, which cross the midline and ascend through the spinothalamic tract to supraspinal targets**,** or

synapse with other cells involved in the rapid, reflexive or sympathetic responses to pain (McQuay, 2009).

3. **Cortical Processing**: Impulses reach the neothalamus via the neospinothalamic tract, and are then relayed to the primary (S_1) and secondary (S_2) somatosensory cortices. This area is responsible for the perception and qualification of the pain stimulus (McQuay, 2009).

1.2.2. The Affective-Motivational Pathway

This pathway processes the subjective, emotional experience of pain, which is critical for driving avoidance behaviour, unpleasantness, memory of past pain, pain-related anxiety etc. (Refsgaard et al, 2016).

Nociceptive impulses travelling from the spinal cord make their way through numerous ascending tracts**,** especially the paleospinothalamic tract, to the medial thalamus, and onward to various cortical and subcortical regions in the brain, that constitute the 'pain matrix' and are involved in emotional and limbic function (McQuay, 2009). Most prominent amongst these are the Cingulate gyrus (CG), the Insula (specifically the anterior insula), the Frontal Cortex (FC), and the Amygdala (McQuay, 2009).

1. **Cingulate Gyrus (CG):** The CG encodes the unpleasantness and aversiveness of pain, mediating the motivation to escape the painful stimulus (Refsgaard et al, 2016). Its activity is strongly correlated with the emotional distress associated with pain.
2. **Insula**: This region integrates sensory, affective, and cognitive information. The Anterior Insula is thought to contribute significantly to pain anticicpation and pain-related anxiety (McQuay, 2009).
3. **Frontal Cortex (FG)**: The region, well known for mediating intelligent thought, is also implicated in the processing of pain, either inducing pain-chronification or providing anti-nociceptive effect via modulatory neural pathways (Ong, Stohler and Herr, 2018).

4. **Amygdala**: A central structure in the limbic system, the amygdala is crucial for processing fear, stress, and anxiety (Lezak et al, 2017). Pain signals project to the amygdala, where persistent noxious input can lead to neuronal hyper-excitability, thereby driving the development of anxiety-like behaviours observed in preclinical models (Parent et al, 2012).

While these structures have been found to amplify pain perception and behavioural responses, impulses originating from these regions and those of the sensory-discriminative component, may serve to reduce pain, acting via descending inhibitory pathways that suppress nociceptive transmission. These descending pathways are themselves modulated by an individual's psychology and emotions, with states like anxiety, depression and stress, limiting descending inhibition and thereby increasing pain sensitivity and intensity (McQuay, 2009).

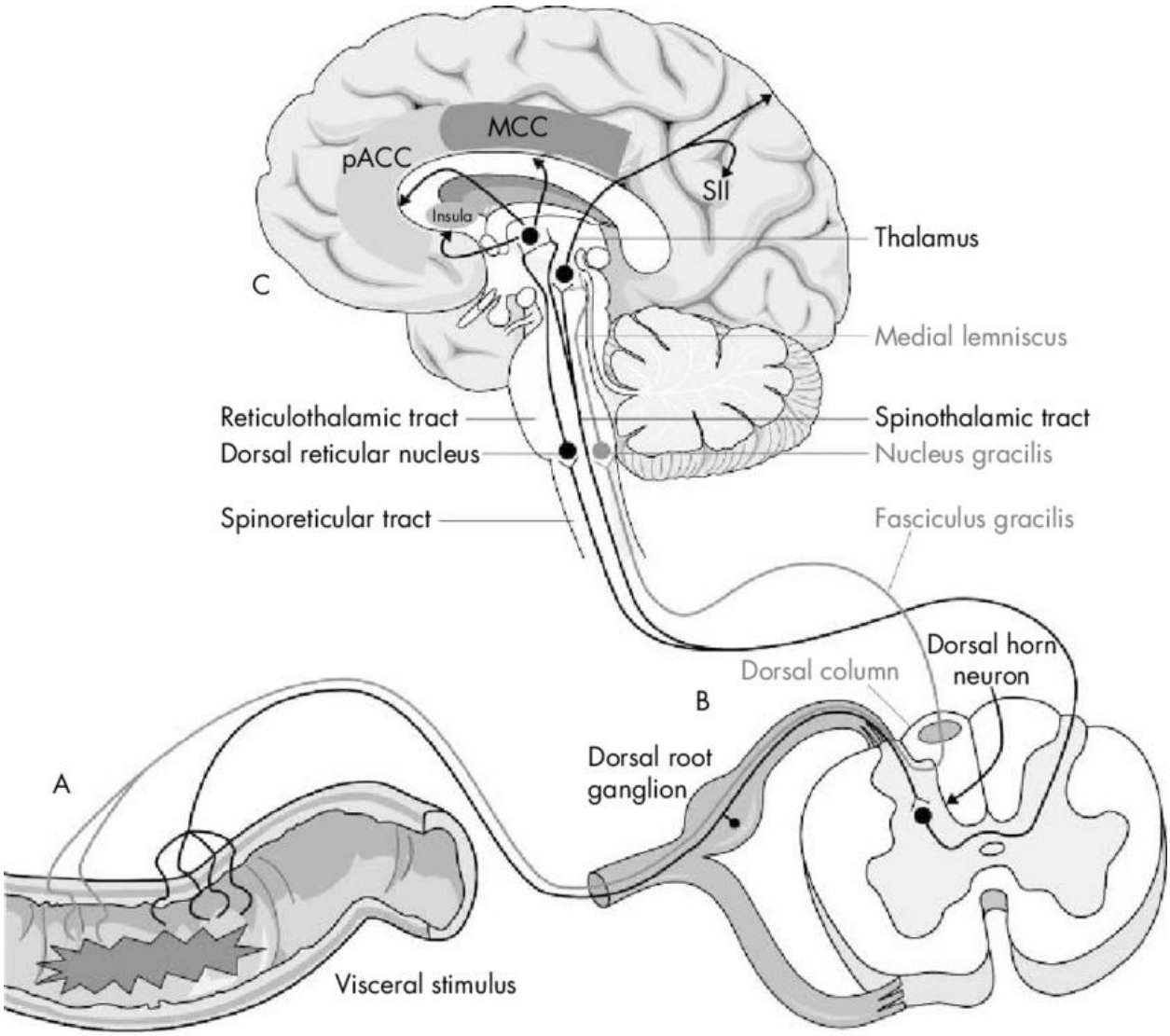

Figure 1.1: Anatomical overview of pain pathways. Noxious stimuli are conveyed from peripheral nociceptors to the dorsal horn through nervous fibers. Second-order spinal neurons send impulses rostrally via the spinothalamic tracts. These cells also activate motor and sympathetic efferents within the spinal cord. Ascending tracts make contacts in the brainstem and midbrain, central gray, and thalamus. Projections are then made with the frontal and limbic cortex. Descending fibers emanating from cortex, hypothalamus, and brainstem project to the spinal cord to modulate pain transmission.

1.2.3. Biochemistry of Nociception

The perception of pain is also linked to a series of biochemical events, which are elicited on tissue injury and progress through various stages of neural processing. These molecular interactions, along with modulation within the central nervous system, mediate pain processing and transmission.

Using a regional and functional framework, a range of substances involved are given below:

1. **Peripheral Activation and Sensitization**: At the instant of injury or bodily insult, damaged cells release arachidonic acid (AA), which is converted to various prostaglandins, potassium (K^+) and hydrogen ions (H^+) which depolarise peripheral nociceptors. These nociceptors also convert other noxious stimuli into calcium ($Ca^{2+)}$ mediated depolarization current. Bradykinin, prostaglandins (PGE_2, PGI_2), serotonin (5-HT), histamine, ATP, and leukotrienes are refered to as *primary inflammatory mediators*, and bind to specific metabotropic receptors to lower activation thresholds and produce peripheral sensitization. These primary mediators go on to stimulate the release of peptides and neurokinins e.g. calcitonin, cholecystokinin, substance P, which promote increased levels of the former via positive feedback (McQuay, 2009).
2. **Immune and Inflammatory Mediation**: Local immune responses amplify this process, with cytokines such as interleukin-1β, interleukin-6, and tumour necrosis factor-α, increasing vascular permeability and excitability of nociceptors. These inflammatory mediators also go on to activate transient receptor potential (TRP) ion channels, generating action potentials in nociceptors, which are conducted to the dorsal horn of the spinal cord (Cocea and Stoica, 2024).
3. **Spinal Transmission**: At the spinal cord, nociceptor terminals release glutamate, which is the principal *fast excitatory transmitter*. It activates AMPA, kainate and consequently voltage-gated NMDA receptors, allowing Ca^{2+} influx which triggers downstream signaling driving central sensitization. Peptides e.g. substance P and calcitonin gene-related protein (CGRP), also released

in this region are implicated in delayed and long-lasting depolarization of second-order dorsal horn neurons (McQuay, 2009).

4. **Inhibitory Modulation**: Counterbalancing excitation of these neurons are γ-aminobutyric acid (GABA) and glycine, which hyperpolarize dorsal-horn neurons via their respective receptors. Also, endogenous opioids (enkephalins, endorphins) act at pre-synaptic and post-synaptic μ-, δ-, and κ-opioid receptors to inhibit neurotransmitter release and reduce responsiveness (Lee and Neumeister, 2020b).
5. **Ascending and Descending Pathways**: Excited second-order neurons project via ascending tracts, and use glutamate and aspartate as their principal transmitters, while being modulated by descending pathways, which inhibit or facilitate transmission, via serotonergic, adrenergic, and opioid activity (McQuay, 2009).

1.3. DRUGS UNDER STUDY: CHEMISTRY AND PHARMACOLOGY

1.3.1. Orphenadrine

Orphenadrine is a centrally acting skeletal muscle relaxant. It was initially developed in the 1940's for the management of Parkinson's disease. Chemically, it is a monomethylated derivative of the first-generation anti-histamine diphenhydramine, with the molecular formula $C_{18}H_{23}NO$. The supplied therapeutic formulation is the racemate of its (R) and (S) enantiomers (PubChem, 2025). Its chemical structure is shown below:

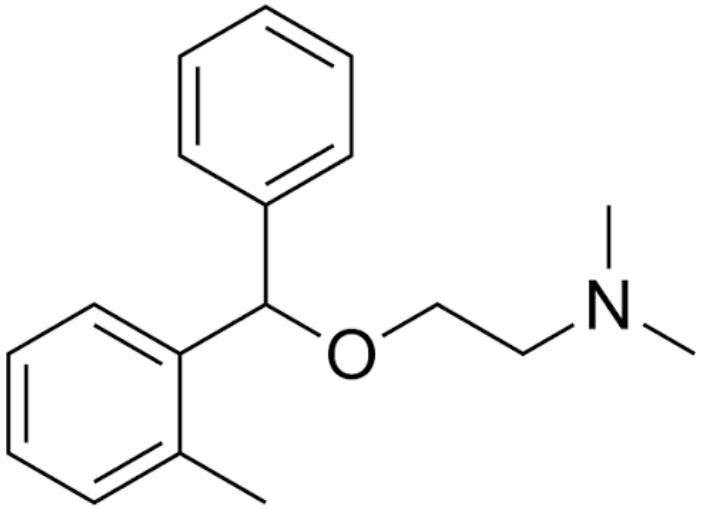

Figure 1.2: Chemical Structure of Orphenadrine

1.3.1.1. Pharmacodynamics

Despite its unclear pharmacodynamic profile, orphenadrine is known to exert its muscle-relaxant and analgesic activity via the following mechanisms:

1. **Anti-cholinergic activity**: Orphenadrine acts as an antagonist on muscarinic cholinergic receptors, with some studies stating that it shows as much as 58% the potency of atropine, leading to its anticholinergic side effects commonly seen in clinical use e.g xerostomia, dry eyes, urinary retention, postural hypotension etc. This activity contributes to its analgesic effect, as

cholinergic activity, mediated in part by opiate receptors in the central nervous system, has been implicated in nociception (Hunskaar & Donnell, 1991; Brunton & Knollman, 2023).

2. **Histamine (H_1) receptor antagonism:** Being an analogue of the common antihistamine, diphenhydramine, orphenadrine also exerts similar activity, inhibiting histaminergic transmission, by exerting blockade on G-protein coupled H_1 receptors, distributed widely throughout the body, particularly in the central nervous system, smooth muscles and immune cells (Hunskaar & Donnell, 1991).
3. **NMDA receptor antagonism**: Orphenadrine also acts at N-methyl-D-Aspartate receptors as an antagonist, albeit with relatively low potency. These receptors are particularly expressed in the central nervous system, and they are known to play a role in pain transmission, facilitating impulse transmission across the spinal cord to supraspinal centers (Kornhuber et al, 1995).
4. **Monoaminergic activity**: Acting as a reuptake inhibitor at norepinephrine receptors in the CNS, orphenadrine is associated increased levels of monoamines at the synapse, which is especially notable at descending inhibitory pathways, where these neurotransmitters serve to inhibit pain transmission and perception (Hunskaar, Berge & Hole, 1985).

1.3.1.2. Pharmacokinetics

Orphenadrine comes in combination with a number of chemical moieties e.g. hydrochloric acid, citric acid etc. The citrate bound formulation is more common in clinical use, and is administered either orally or parenterally. When used via the oral route, it is readily absorbed, primarily at the small intestine, with a bioavailability of 95% and a time to peak plasma concentration of 2 -4 hours. It is widely distributed, most probably crosses the blood brain barrier, and is extensively metabolized in the liver by the cytochrome P450 system. Its metabolites are primarily excreted in the urine, with its elimination half-life ($t_{1/2}$) varying between 13.2 hours to 20.1 hours (Labout *et al.*, 1982).

1.3.1.3. Indications

Orphenadrine is indicated for use in conditions involving skeletal muscle spasms, such as lower back pain, and acute painful musculoskeletal conditions. It is also useful in cases of traumatic injury such as in accidents, or whiplash. In certain cases, it may be prescribed for the management of insomnia (Brunton & Knollman, 2023).

1.3.1.4. Safety Profile

Common side effects associated with the use of orphenadrine include anticholinergic and antihistaminergic effects, such as dry mouth, nausea, dysuria, constipation, drowsiness, headache, confusion etc. (Hunskaar & Donnell, 1991). More severe adverse effects include increased intraocular pressure, hallucinations (NMDA antagonism is implicated), tachycardia, syncope, heart palpitations etc. Rarely, orphenadrine use has been associated with increased occurrence of aplastic anaemia, although some sources say no definite cause-effect relationship has been established. Urticaria, and other pruritic dermatoses have been reported and anaphylactic reactions have been reported to occur following orphenadrine injection. Its side and adverse effects can be potentiated on concomitant use with other agents with similar mechanisms of action, e.g. other anti-cholinergics, anti-histamines, NMDA-associated psychedelics, anti-depressants etc (Medicis Pharmaceutical Corporation, 2012).

1.3.1.5. Contraindications

Orphenadrine is avoided in cases of known hypersensitivity to the drug, or any components of the drug formulation. It is also contraindicated in certain disease conditions such as glaucoma, gastrointestinal obstruction, stenosing peptic ulcers, prostatic hypertrophy, urinary retention, cardiomyopathy and myasthenia gravis. Special consideration is given in use in patients with renal, hepatic or cardiac impairment, elderly patients, children, pregnant women, lactating mothers and in cases where addiction is likely (Medicis Pharmaceutical Corporation, 2012).

1.3.2. Diclofenac

Diclofenac is a nonsteroidal anti inflammatory drug (NSAID) belonging to the category of phenylacetic acid derivatives, and is commonly prescribed for its potent analgesic, antipyretic, and anti inflammatory activities (Alfaro and Davis, 2023). Its systematic IUPAC designation is 2-[2-(2, 6-dichloroanilino)phenyl]acetic acid with a molecular formula of $C_{14}H_{11}Cl_2NO_2$ (PubChem, 2025). At physiological pH, the drug mainly exists in its ionized carboxylate state, which influences its absorption, distribution and interaction with cellular membranes.

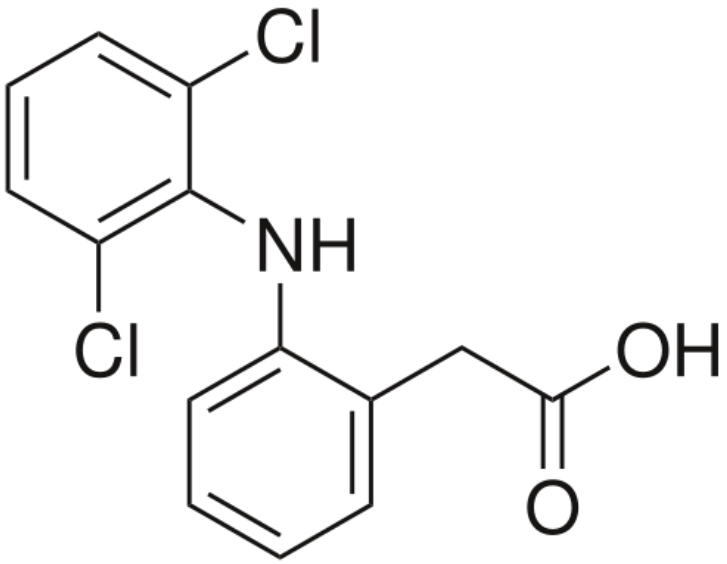

Figure 1.3: Chemical Structure of Diclofenac

1.3.2.1. Pharmacodynamics

Diclofenac exerts its therapeutic effect via its activity against cyclooxygenase enzymes in vivo. These enzymes, foremost in the biosynthesis of prostaglandins, are involved in the conversion of arachidonic acid to the unstable intermediates PGG_2 and PGH_2 which undergo further transformation to yield the prostanoids, thromboxane A_2, and a variety of prostaglandins. COX-1, expressed constitutively in most cells, is the dominant source of prostanoids for housekeeping functions, such as hemostasis. Conversely,

COX-2, induced by cytokines, shear stress, and tumor promoters, is the more important source of prostanoid formation in inflammation. These prostanoids play a crucial role as primary inflammatory mediators, leading to a cascade of events that characterise a full inflammatory response. Diclofenac inhibits the activity of both these isoforms, limiting the production of prostanoids. Although it was not developed as a selective COX-2 inhibitor, the selectivity of diclofenac resembles that of celecoxib, which specifically targets COX-2. Diclofenac also shows comparative advantage over other NSAIDS, having substantially greater potency than its counterparts (Brunton & Knollman, 2023).

1.3.2.2. Pharmacokinetics

Diclofenac is rapidly absorbed following oral administration, extensively protein bound, and has a plasma half-life of 1 to 2 hours. This short half-life necessitates doses higher than that needed to inhibit COX-2 fully at peak plasma concentrations, to afford sustained COX inhibition throughout the dosing interval. Thus, both COX isoforms are inhibited for the first phase of the dosing interval, due to the higher concentrations of the drug in systemic circulation. However, as plasma levels decrease over the course of the dosing interval, diclofenac displays more selective inhibition on the COX-2 isoform. There is a substantial first-pass effect, such that only 50% of an oral dose administered reaches systemic circulation. The drug accumulates in synovial fluid following oral administration, which may explain why the duration of its therapeutic effect is much longer than would be predicted based on its plasma half-life. Diclofenac is metabolized in the liver by a member of the CYP2C subfamily to 4-hydroxydiclofenac, the principal metabolite, and other hydroxylated forms; after glucuronidation and sulfation, the metabolites are excreted in the urine (65%) and bile (35%). Multiple oral and parenteral formulations are available, providing a wide range of pharmacokinetic variety and bioavailability profiles (Brunton & Knollman, 2023).

1.3.2.3. Indications

Diclofenac is commonly used for the long-term symptomatic treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, primary dysmenorrhea, and acute migraine. It is also used in the management of pain and inflammation associated with minor injuries, and even surgical procedures such as cataract extraction or corneal refractive surgery. Diclofenac is also available in combination with misoprostol, a PGE1 analogue, to reduce the risk of gastrointestinal ulcers (Brunton & Knollman, 2023).

1.3.2.4. Safety Profile

Diclofenac produces side effects (particularly gastrointestinal) in about 20% of patients. The incidence of serious GI adverse effects, hypertension, and myocardial infarction is similar to that observed with the COX-2–selective inhibitors (Cannon et al, 2006). Hypersensitivity reactions have occurred following topical application and systemic administration. Severe liver injury and hepatotoxicity has also been reported in, but are far less common. Other adverse events associated with diclofenac use include CNS effects, rashes, fluid retention, edema, and renal function impairment. Diclofenac is extensively metabolized and one metabolite, 4′-hydroxydiclofenac, can form reactive benzoquinone imines (similar to acetaminophen's metabolite NAPQI) that deplete hepatic glutathione. UGT2B7 is the primary catalyst in the formation of another highly reactive metabolite, diclofenac acyl glucuronide (King et al, 2001).

1.3.2.5. Contraindications

Diclofenac is not recommended for children, nursing mothers, or pregnant women. It is contraindicated in patients with known hypersensitivity, history of gastrointestinal bleeding or perforation associated with diclofenac use, bleeding disorders, and fluid imbalance.

1.4. PRECLINICAL MODELS OF ACUTE PAIN IN RODENTS

In an effort to reflect and measure the total experience of pain as found in humans, a vast array of analgesiometric models have been developed for use on laboratory animals, especially rodents. It is worthy of note that most these models, view pain-related behaviour as a reflexive response to applied stimulus, studying mainly the hypersensitivity (allodynia and hyperalgesia) associated with painful stimuli. They are commonly termed evoked reflexive assays, as pain response is "evoked" as a result of external stimulus (Larson, Wilcox & Fairbanks, 2019).

These assays are commonly classified into mechanical, thermal, chemical or electrical types (Mogil, 2009), based on the nature of the noxious stimuli applied. The following section discusses these categories and some common examples under each.

1. **Mechanical assays**: These assays assess the sensitivity of the animals under study, to pressure or touch. Commonly used are the von Frey and grip force models. The von Frey model applies pressure on the plantar surface of the paw, up until a paw withdrawal threshold is reached, the point at which further pressure is intolerable. The grip force assay is commonly used in the context of lower back pain. Here, mice grasp onto a bar, and are gently pulled back by their tails until they release the bar. These assays measure pain in terms of maximum grams of force tolerated or exerted, measured by means of an analgesiometer (Larson, Wilcox & Fairbanks, 2019).
2. **Thermal assays**: Thermal assays are highly common for screening analgesic activity, especially for compounds targeting the central nervous system, such as opiods. These models mostly measure pain response in terms of latency to withdraw from the noxious stimuli. They include the popular tail flick and hot water immersion models, all of which apply heat to a region of the animal's skin (usually its tail) to evoke a reflexive movement of the tail away from the heat

source (Singh, Bilwal & Godara, 2019). Less common are the models assessing cold allodynia—the activation of cold nociceptor responses by normally non-painful cold temperatures—including the cold water tail flick assay or the use of chemicals such as menthol or eucalyptol, which act at TRP receptors to elicit cold sensations (Larson, Wilcox & Fairbanks, 2019).

3. **Chemical (and Inflammatory) assays:** These assays induce a longer-lasting, tonic pain state often involving behavioural responses that require supraspinal processing, inducing inflammation using a wide variety of chemical insults. Common agents used for this purpose include formalin, capsaicin, collagen, carrageenan, bacterial lipopolysaccharide and even suspensions of whole microbes as seen in the complete Freud's adjuvant. These agents elicit inflammation indirectly by triggering the release of primary inflammatory mediators, which go on to elicit local and even neural responses, and consequent pain-related behaviours, such as paw-licking, writhing, joint-pain etc. (Mogil, 2009). The model used in this research i.e. the acetic acid induced mouse writhing model, also falls under this category.
4. **Electrical Assays**: These elicit pain by delivering a quantifiable electrical stimulus directly to a nerve or tissue, often the tail (tail-shock model), the skin (as seen in foot shock), or to the dental pulp (tooth pain model) (Yam et al, 2020).

Other examples of evoked pain assays are the neuropathic assays, where nerve damage is induced in a bid to reflect the conditions found in neuropathic pain, and painful disease assays, which attempt to mimic disease, injury or pathophysiologic states associated with pain, e.g. assays of burn-related pain, surgically induced osteoarthritic pain, labour pains etc. (Mogil, 2009).

While evoked assays remain the gold standard for confirming the establishment of pain states, the use of the newer spontaneous behaviour measures in the assay of pain present a more holistic method of capturing the more complex and clinically relevant aspects of the pain experience, particularly ongoing,

non-evoked pain and its associated affective (emotional) component (Whittaker & Howarth, 2014; Refsgaard et al, 2016).

1.5. BEHAVIOURAL ASSAYS OF ANXIETY IN RODENTS

These refer to specific procedures, paradigms or environments used to induce quantifiable anxiety-like behaviour, similar to the natural adaptive behavioural response (Cryan & Sweeney, 2011). Assays specific for anxiety-like behaviours serve to provide insight into the nature of common anxiety disorders and anxiety as a co-morbidity with other conditions.

These assays are commonly classified into two categories: those based on unconditioned behaviours, and others centred around conditioned/learned behaviours (Rodgers, 1997; Harro, 2017).

Unconditioned Behavioural Assays: These assays rely on the rodent's innate or unlearned, spontaneous reactions to a novel or potentially threatening stimulus or environment. Anxiety-like behaviour is inferred from the animal's aversiveness to continued exposure to stimuli. Examples of unconditioned assays include:

1. **Elevated Plus (or Y) Maze**: Animals are placed on an elevated plus-shaped (or Y-shaped) maze consisting of open and enclosed segments connected by a central connecting area. The open segments of the maze are considered to be more aversive than the closed arms, and the number of entries and time spent in any of the segments is recorded (Rodgers, 1997).
2. **Hole Board Experiment**: Rats or mice are placed on an opaque board, with several regularly spaced perforations. Exploratory behaviour (number of holes visited, number of head dips into holes), locomotor activity, as well as a number of ethological parameters can be measured (Cryan & Sweeney, 2011).

3. **Ultrasonic Vocalizations**: Mouse pups are separated from their mothers and the number and frequency of ultrasonic distress calls is recorded. The number of ultrasonic calls emitted from separated pups is used as a measure of anxiety (Harro, 2017).

Conditioned Behavioural Assays: Conditioned assays require the animal to learn an association between an initially neutral or innocuous stimulus (or environment) and an aversive outcome, thus generating a learned fear or anxiety-like state. Examples include:

1. **Conditioned Emotional Response**: Animals are trained to associate a specific operant, feeding or drinking behaviour with unpleasant stimuli. This association results in a decrease in the performance of the said behaviour, and prompts learned anxiety. E.g. Vogel's Punished Drinking (Cryan & Sweeney, 2011).
2. **Pavlovian Fear Conditioning**: Animals are trained to associate a particular contextual or cue-related stimulus (CS) with an unpleasant stimulus such as an electric foot shock, acoustic stimulus or an aversive odour (US). The animal then learns to display an observable startle or freezing response to the CS independent of the presence of the US (Cryan & Sweeney, 2011).
3. **Novelty-induced Hypophagia**: Animals are trained to consume a desirable food (e.g. sweetened milk) before being presented with this same item in a novel environment. Hesitation on the part of the animal to consume the food in the novel environment is regarded as a measure of both anxiety and anhedonia. (Haller & Alicki, 2012).

Despite concerns as regards the external validity of these assays being raised by many observers, they remain of practical utility in various preclinical settings, serving as both screening tools in the search for novel anxiogenic or anxiolytic agents and as simulations in studies for investigating the underlying mechanisms of disease progression (Hånell & Marklund, 2014).

1.6. BIOCHEMICAL CORRELATES: OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between levels of pro-oxidants and anxi-oxidants in-vivo, arising due to the relative overproduction of reactive oxygen species (ROS), either free radicals e.g. superoxide anion ($O_2•^-$), hydroxyl radical (OH•), hydroperoxyl radical ($H_2O•$), or non-radical forms e.g. hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) etc. Cells in living systems are constantly under oxidative attack from ROS, which in normal conditions, play a crucial role as second messengers in cell signaling and metabolic pathways, but are linked to cell damage and death at toxic levels, with increased production induced by a number of factors e.g. inflammation, radiation, environmental factors etc. The body's intrinsic antioxidant system, or its total antioxidant capacity, made up of enzymatic and non-enzymatic antioxidants, serves to keep oxidative stress in check, maintaining homeostatic balance (Tejchman, Kotfis & Sienko, 2021)

Oxidative imbalance is a well-known factor contributing to various adverse events and conditions including atherosclerosis, cardiovascular complications, Alzheimer's disease, glaucoma etc. In a study on the diagnostic value of biomarkers of oxidative stress in patients suffering from generalised anxiety disorders, a link was established between oxidative stress and the progression of a number of conditions, namely obsessive compulsive disorder, social phobia and post-traumatic stress disorder (Findikli et al, 2018). Other studies support this relationship, linking anxiety to over expression of pro-oxidants or lowered anti-oxidant capacity in the brain and periphery (Bouayed, Rammal & Soulimani, 2009; Smaga et al, 2014). Oxidative stress was also suggested to play a role in the etiopathogenesis of inflammatory and non-inflammatory acute and chronic back pain (Inanir et al, 2013).

In the evaluation of oxidative stress levels, certain biochemical compounds are used as an indicator of the degree of pro-oxidant attack. ROS are hardly used directly as biomarkers due to their short half-lives; rather their by-products from reactions with endogenous molecules serve this purpose, leaving a

unique, quantifiable, chemical "fingerprint". A number of criteria are important in the choice of a compound to be used as a biomarker, including its use in detection of a major part of total ongoing oxidative damage, provision of coherent laboratory assays, consistency of measured results, stability, independence from dietary changes etc (Tejchman, Kotfis & Sienko, 2021).

Although there is no ideal biomarker, many suffice for the purpose they are intended. Common examples of these are:

1. **Catalase (CAT)**: Catalase is a tetrameric heme enzyme found mainly in peroxisomes, that rapidly decomposes hydrogen peroxide into water and oxygen. Its exceptional turnover rate makes it one of the fastest acting enzymes. Its activity is altered in metabolic stress (Tejchman, Kotfis & Sienko, 2021).
2. **Glutathione (GSH)**: A tripeptide (γ-glutamyl-cysteinyl-glycine) that acts as a universal cellular redox buffer. It donates electrons to help detoxify reactive species, and also serves to maintain protein thiols in their reduced form. Its ratio to oxidized GSSG is an important indicator of cell stress (Frijhoff et al, 2015).
3. **Glutathione peroxidase (GPx)**: GPx enzymes are selenium-containing peroxidases that make use of reduced glutathione (GSH) to detoxify lipid hydroperoxides and H_2O_2. Dependent on selenium levels, they are sensitive to dietary deficiency (Tejchman, Kotfis & Sienko, 2021).
4. **Malondialdehyde (MDA)**: A small dialdehyde ($CH_2(CHO)_2$) that is generated by oxidation of polyunsaturated fatty acid. It forms covalent adducts with proteins (advanced lipoxidation end-products, ALEs) and DNA, making it both a marker and mediator of oxidative injury. (Ho et al, 2013).

5. **Dityrosine (DiY)**: This is a biphenyl compound formed by oxidative coupling of two tyrosine residues. Its fluorescence and stability make it a sensitive indicator of protein crosslinking and consequently, oxidative stress (Tejchman, Kotfis & Sienko, 2021).

1.7. MALONDIALDEHYDE AS A BIOMARKER OF OXIDATIVE STRESS IN ACUTE PAIN AND ANXIETY

Malondialdehyde (MDA) as previously described, is a small biomolecule generated in vivo via peroxidation of polyunsaturated fatty acids having two or more methylene-interrupted double bonds. MDA can also be generated in vivo from various prostaglandins via certain enzyme mediated processes, e.g. biosynthesis of thromboxane A_2, which leads to formation of MDA and 12(S)-hydroxy-8,10(E,E)-heptadecadienoic acid (HHT) as secondary products (Rio, Stewart & Pellegrini, 2005).

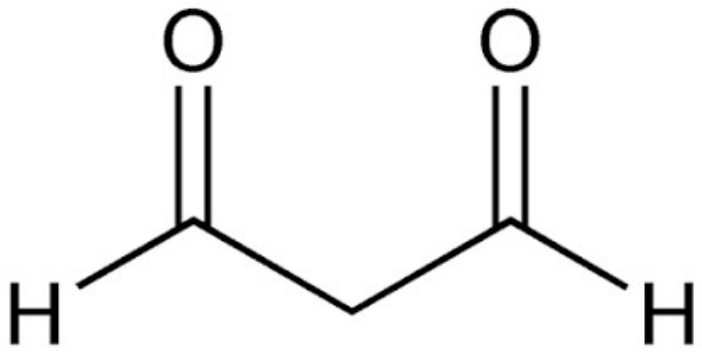

Figure 1.4: Chemical Structure of Malondialdehyde

Under normal physiological conditions, MDA exists as an enolate anion with low chemical activity. Despite this, it is able to form numerous adducts and participate in several reactions in vivo, a characteristic which precedes its role as a mediator of oxidative damage and a biomarker of oxidative stress. MDA is known for its extensive toxicity profile, especially its mutagenic and carcinogenic effects, which occur as a result of its interaction with several molecules in vivo, and lead to formation of

interstrand cross-links in DNA, formation of DNA-protein cross-links, formation of DNA-histone cross-links etc. (Rio, Stewart & Pellegrini, 2005).

A number of studies have established a link between anxiety and lipid peroxidation in the CNS, evidenced by increased malondialdehyde levels observed as mice and wistar rats were exposed to various stressors including restraint, acute immobilisation and hypoxia. It was also found that biochemical manipulations which affected lipid metabolism directly or indirectly through changes in oxidative balance, also led to either anxiogenic or anxiolytic effects, with substances like L-buthionine-(S,R)-sulfoximine (an inducer of oxidative stress) inducing anxiety-like behaviours, supplementation with vitamin A or deficit of vitamin E, both of which enhance lipid oxidation, also exerting anxiogenic effect (Smaga et al, 2014).

Further evidence to the fact was brought to light by the activity of common anxiolytic drugs on MDA levels in vivo. Agents such as diazepam, alprazolam, zolpidem (all $GABA_A$ agonists), and buspiron (a serotonin receptor agonist), all reversed lipid peroxidation resulting from induction of anxiety, leading to lowered MDA levels (Smaga et al, 2014; Matsumoto et al, 1999). The reverse case was also observed, with certain antioxidants e.g. tempol, sesamol, and N-acetylcysteine, also possessing marked anxiolytic effects in rodents (Smaga et al, 2014).

A relationship has also been found to exist between lipid peroxidation and consequent MDA levels, acute pain, and inflammation in vivo, as elevated MDA concentrations have been associated with increased inflammation and pain intensity, suggesting that oxidative degradation in vivo may play a role in sensitising pain pathways. Plasma MDA has been shown to rise, in early stages of acute inflammatory conditions such as pancreatitis, correlating with pain severity and tissue injury (Hernández et al., 2006), while higher MDA levels also correspond to greater pain intensity in conditions like primary dysmenorrhea (Ocktariyana et al, 2023). Postoperative increases in MDA have further been linked with

markers of systemic inflammation, including C-reactive protein, suggesting that the oxidative response to surgical trauma contributes to pain and recovery outcomes (Vujović et al, 2023). However, not all acute or subacute pain states show systemic oxidative changes; for instance, no significant elevation in circulating MDA was found in short-term complex regional pain syndrome, implying that oxidative stress in some pain syndromes may be localized rather than systemic, and that the diagnostic applicability of MDA as a biomarker of oxidative stress accompanying acute pain and inflammation, depends on the nature and site of the underlying pathology (Fischer et al, 2013).

1.8. JUSTIFICATION OF THE STUDY

The burden of understanding and managing anxiety in the context of acute pain, has persisted and still presents a significant challenge to researchers and clinicians alike (Zhang et al, 2024; Michaelides & Zis, 2019). Although a relationship between anxiety and acute pain has been well established, with anxiety-like behaviours being associated with an increased pain severity, and the experience of acute pain itself leading to dysregulation of affective states (Michaelides & Zis, 2019), there remains significant potential for deeper insight through extensive research (Zhang et al, 2024).

The choice of the drugs under study i.e. diclofenac and orphenadrine, facilitates direct comparison between two distinct analgesic mechanisms, i.e. the peripheral mediation of pain, via inhibition of cyclooxygenase (COX) enzymes, affecting solely the sensory-discriminative component of pain, and the central inhibition of pain conduction and positive modulation, regulating both nociception and possibly the affective-motivational component of the pain experience. Evaluating the combination of these two drugs also allows us to test for potential synergisms, and would reflect a more holistic experimental approach.

This study necessitates a multifaceted assay system, providing multiple essential perspectives. This system includes an acute pain assay i.e. the acetic-acid induced mouse writhing test, which confirms the analgesic efficacy of the drugs against exposure to a noxious stimulus, a behavioural assay, the elevated plus maze, which directly measures the anxiotropic effect of the drugs by measuring post-exposure anxiety-like behavior, and a biochemical assay, which evaluates oxidative stress via measurement of malondialdehyde levels, to provide insight into likely mechanisms of action.

1.9. AIMS AND OBJECTIVES

This primary aim of this study was to evaluate the anxiotropic profile and existing synergisms between orphenadrine and diclofenac on behavioural responses elicited in acute pain.

Specific objectives include:

i. comparing the analgesic and anxiotropic effects of diclofenac, orphenadrine, and their combination in Swiss albino mice, using the acetic-acid induced mouse writhing assay and the elevated plus maze.
ii. investigating changes in oxidative stress, measured by malondialdehyde (MDA) levels, and their association with the observed behavioural effects.

CHAPTER TWO

2.0. MATERIALS AND METHODS

This chapter describes the experimental approach for the evaluation of the analgesic and anxiotropic effects of orphenadrine, alone and in combination with diclofenac in Swiss albino mice, outlining the apparatus, chemicals, methods of handling for the experimental animals, procedures of the assays on acute pain (acetic acid induced mouse writhing), anxiety-like behaviour (elevated plus maze), oxidative stress (TBA derivatisation), and statistical methods employed for data analysis.

2.1. MATERIALS

2.1.1. Drugs and chemicals

5mg/ml Diclofenac sodium injection, Orphenadrine citrate (Norflex®), Normal saline (Bioflex®), Acetic acid solution (0.6% v/v in saline), Chloroform (BDH Chemicals, England®), Distilled water, Reagents for Malondialdehyde assay, 10% buffered formaldehyde solution.

2.1.2. Apparatus

Syringes (1mL and 5mL) and hypodermic needles, beakers, weighing balance, orogastric tubes, measuring cylinders, universal sample bottles, hand gloves, coloured markers, cotton wool, masking tape, timer/stopwatch, elevated Plus Maze apparatus, non-toxic animal marking kit, transparent observation chamber,

2.1.3. Experimental animals

Male and Female Albino mice weighing 18-35 grams were bred in the facilities of the Department of Pharmacology at the University of Benin in Benin City, Edo State, Nigeria, and used in this investigation. The mice were housed at 27 ± 2ºC in a controlled laboratory setting with a 12-hour light/dark cycle.

They also had unrestricted access to commercial food and water, *ad libitum*. The study followed internationally accepted standards for the usage and ethical treatment of laboratory animals, as stated in the European Community's guidelines **(EEC Directive of 1986; 86/609/EEC).**

2.0. METHODS

2.0.1. Body Weight

The body weight of each mouse was carefully determined and recorded on the first day of the study.

2.0.2. Grouping and Drug Administration

The animals were assigned into four (4) groups of six (n = 6) mice each:

i. Group 1 (Control): Received normal saline (10 mL/kg, orally).

ii. Group 2 (Diclofenac): Received diclofenac sodium (50 mg/kg, intraperitoneally).

iii. Group 3 (Orphenadrine): Received orphenadrine citrate (25 mg/kg, orally).

iv. Group 4 (Orphenadrine-Diclofenac Combination): Received a combination of orphenadrine citrate (25 mg/kg, orally) and diclofenac sodium (50 mg/kg, intraperitoneally).

Each mouse was marked on the tail using a non-toxic coloured marker to ease recognition.

2.0.3. Acetic Acid Induced Mouse Writhing Test

Peripheral analgesic activity was assessed using the acetic acid-induced writhing model. Thirty (30) minutes after administration of the respective agents, each mouse received pre-calculated volumes of 0.6 %v/v acetic acid (10 mL/kg, i.p). Immediately after injection, animals were placed individually in transparent observation cages, and the number of abdominal constrictions (“writhes”) was recorded for

thirty (30) minutes, with counts taken and subdivided under each 5-minute interval of this observation period (Singh, Bilwal & Godara, 2019).

2.2.4. Elevated Plus Maze (EPM) Test

This was carried out twenty-four(24) hours drug administration and acute pain assays. In the procedure, each mouse was placed at the center of the maze facing an open arm and allowed to explore freely for five (5) minutes.

The following parameters were recorded:

i. The number of entries into either the open or closed arms (entry is defined as all four paws passing into an arm).
ii. Cumulative time (in seconds) spent in open and closed arms (Walf & Frye, 2007).

2.2.5. Sample Collection

After completion of behavioral assessments, all animals were humanely sacrificed while under chloroform-induced anesthesia. The brain, kidney and liver were carefully excised and examined for any visible abnormalities or lesions. The tissues were then preserved in 10% buffered formaldehyde solution for subsequent biochemical assays.

2.2.6. Biochemical Assay

Malondialdehyde (MDA) levels in brain tissue were quantified using a thiobarbituric acid (TBA) derivatization method followed by high-performance liquid chromatography (HPLC). Homogenised brain tissues were acidified and reacted with TBA under heat to form the MDA–TBA adduct. The reaction mixture was then cooled, centrifuged, and the supernatant fluid injected into an HPLC system having a fluorescence or UV detector. Separation of the MDA–TBA adduct from interfering compounds was achieved using a reversed-phase C18 column and an appropriate mobile phase. MDA

concentrations were determined by comparisson with a standard calibration curve prepared from known MDA solutions (Findikli et al, 2018).

2.2.7. Data Analysis

All data were expressed as Mean ± Standard Error of the Mean (SEM). Statistical analyses were performed using GraphPad Prism 8.0.1 (San Diego, California, USA). Data from the acetic-acid induced mouse writhing test were analyzed using one-way Analysis of Variance (ANOVA). Data from the EPM test (baseline vs. post-treatment) were analyzed using two-way ANOVA with treatment and time as factors, followed by appropriate post-hoc analysis. Correlations between behavioral and biochemical outcomes were also assessed. Differences were considered statistically significant at $p < 0.05$.

CHAPTER THREE

3.0. RESULTS

3.1. EFFECT OF DRUG TREATMENTS ON WRITHING RESPONSE

The acetic acid induced mouse-writhing model used to evaluate the efficacy of analgesics and correlates a reduction in the number of "writhes" (abdominal constrictions) to a reduction in pain severity. The control group exhibited a high number of writhes, peaking between 10 and 15 minutes. Treatment with orphenadrine (25 mg/kg) substantially reduced the number of writhes compared to the control. Diclofenac (50 mg/kg) and the orphenadrine-diclofenac combination demonstrated a more potent analgesic effect, almost completely abolishing the writhing response. A significant difference was observed between the orphenadrine group and the diclofenac group ($p < 0.001$, $p < 0.01$), indicating stronger analgesic activity of diclofenac in this model.

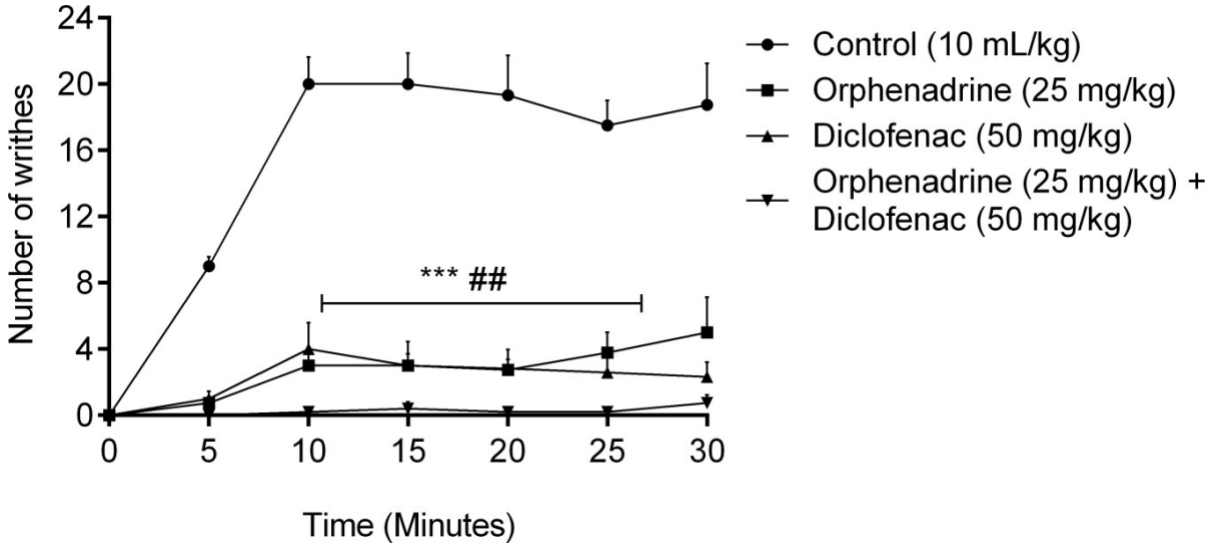

Figure 3.1. Writhing response over thirty (30) minutes. *** indicates a significant difference from the orphenadrine group ($p < 0.001$). ## indicates a significant difference from the orphenadrine group ($p < 0.01$).

3.2. EFFECT OF DRUG TREATMENTS ON MDA LEVELS

Malondialdehyde **(MDA)** is a widely used biomarker for measuring oxidative stress and the resulting lipid peroxidation in tissue. After the treatment period, it was observed that the administration of orphenadrine (25 mg/kg) was associated with increased MDA levels in the brain compared to the control group ($p < 0.0001$). Similarly, diclofenac (50 mg/kg) administration was also linked to a significant increase in MDA levels compared to the control group ($p < 0.001$). The combination of orphenadrine and diclofenac resulted in MDA levels that were lower than those found in either drug administered alone but remained elevated compared to the control group

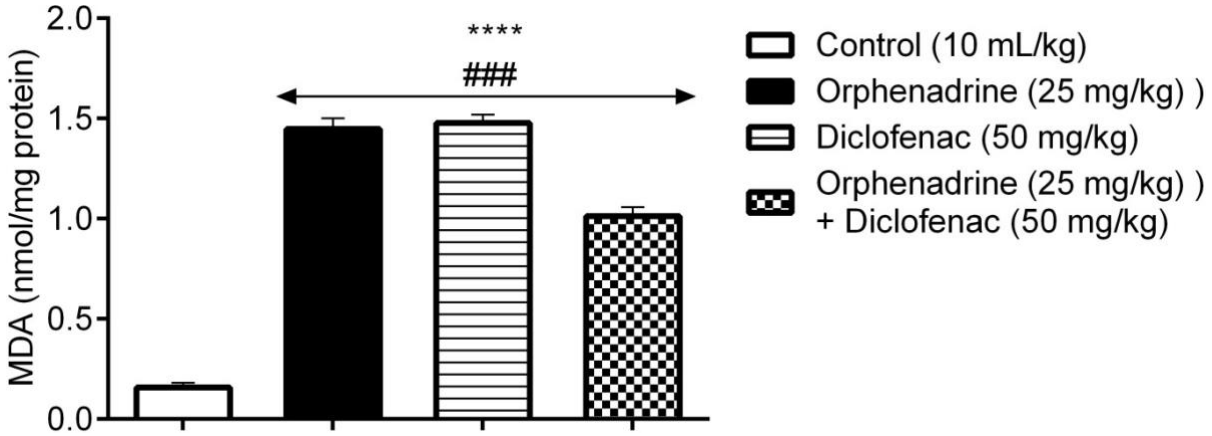

Figure 3.2. Malondialdehyde levels across treatment groups in mmol/mg protein. **** indicates a significant difference from the control group ($p < 0.0001$). ### indicates a significant difference from the control group ($p < 0.001$)

3.3. EFFECT OF DRUG TREATMENTS ON TIME SPENT IN ARMS OF EPM

The elevated plus maze (EPM) is a behavioral assay used to assess anxiety states. In this assay, preference for the closed arms and avoidance of the open arms is indicative of anxiety-like behaviour. It was observed that mice in the control group spent significantly more time in the open arm compared to the groups treated with orphenadrine (25 mg/kg), diclofenac (50 mg/kg), or the orphenadrine-diclofenac combination ($p < 0.001$). Conversely, in the closed arm, all three treatment groups (orphenadrine, diclofenac, and the combination) spent slightly more time compared to the control group ($p < 0.001$). This behavioral shift suggests a strong anxiogenic effect associated with the drug treatments.

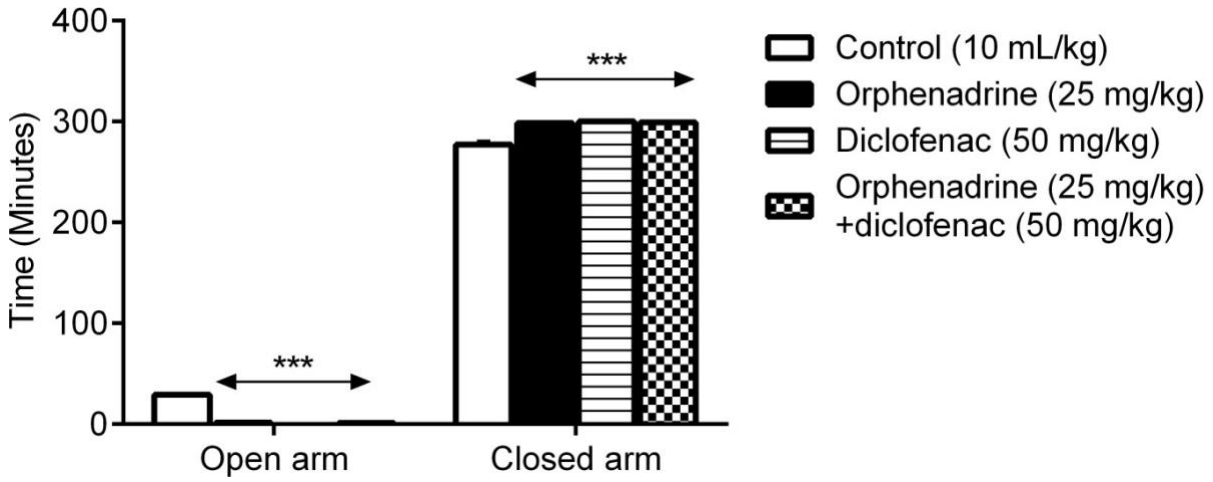

Figure 3.3. Time in seconds spent in the open and closed arms of the EPM. *** indicates a significant difference from the control group ($p < 0.001$).

3.4. EFFECT OF DRUG TREATMENTS ON NUMBER OF ENTRIES INTO ARMS OF EPM

The total number of entries into the arms of the EPM is often used as a measure of exploratory drive and locomotor activity, both associated with lower anxiety levels. For the open arm, entries were lower than those for the closed arm overall, with the control group showing the highest number of open arm entries. The analysis of closed arm entries revealed a significant reduction in the orphenadrine (25 mg/kg) group compared to the control group ($p < 0.01$), suggesting an decrease in exploratory drive and locomotor activity. In contrast, the diclofenac (50 mg/kg) and orphenadrine-diclofenac combination groups both showed a significantly higher number of entries compared to the orphenadrine-only group ($p <0.01$), indicating that their activity was not impaired and was similar to that of the control group.

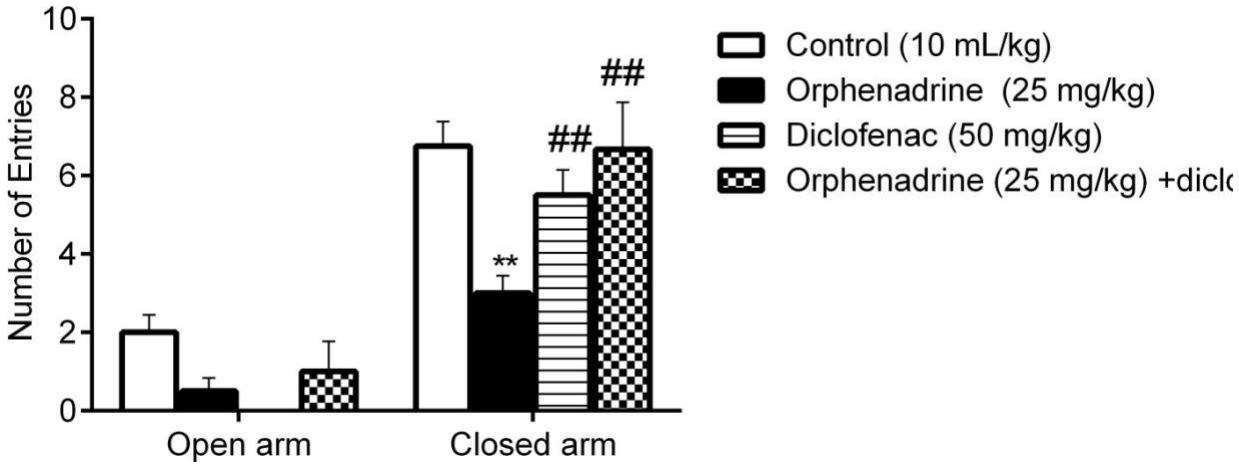

Figure 3.4. Number of entries into the open and closed arms of the EPM. ** indicates a significant difference from the control group ($p < 0.01$). ## indicates a significant difference from the orphenadrine group ($p < 0.01$).

3.5. BODY WEIGHT

The body weight of each mouse was measured and recorded using an electronic weighing balance before the commencement of the study. The mice were then allocated into groups according to specified weight categories, after ensuring that no mouse fell short of a threshold weight of 18.0g. The weights are presented in Table 3.1 through 3.4

CHAPTER FOUR

4.0. DISCUSSION

The experience of acute pain is a complex phenomenon having two major components, i.e. the sensory component, which involves nociception, inflammation, consequent oxidative stress etc., and an affective component defined by psychological states such as anxiety, depression, anger, fear and their proceeding behavioural responses. Despite this, conventional clinical management of acute pain has been mainly focused on physiological mediation, either by mitigating exposure to noxious stimuli, alleviating associated inflammation or blocking the transmission and processing of noxious signals. These limited biomedical interventions, while useful, fail to acknowledge the involvement of psychological and cognitive factors which influence the total pain experience by increasing pain intensity via inhibition of descending pain pathways and lowering the threshold for central sensitization, while being influenced themselves via cortical and biochemical mechanisms (McQuay, 2009).

The development of more holistic alternatives for the management of acute pain requires deeper insight into this bidirectional relationship, which stands at the core of this study, as we seek to evaluate the behavioural and anxiotropic effects of two common analgesics with differing modes of action i.e. orphenadrine, a centrally acting muscle relaxant having anticholinergic, antihistaminergic and putative NMDA-modulatory effects, and diclofenac, a non-steroidal anti-inflammatory drug, suppressing COX-mediated synthesis of prostaglandins and nociceptor sensitization. The combination of these two agents was also evaluated alongside to assess potential interactions and synergisisms.

Also, given increasing evidence that oxidative imbalance and consequent oxidative stress play a crucial role in both the processing of noxious stimuli and anxiety states, this study also includes a biochemical evaluation of oxidative stress levels, using malondialdehyde, a product of lipid peroxidation, as a

biomarker, in a bid to gain insight into the progression of the underlying condition and elucidate the mechanistic basis of drug activity.

The study began with the investigation of the analgesic effects of the drugs under study in comparison to normal saline (a physiological salt solution), which also served as a negative control throughout the experiment. This was done using the acetic-acid induced mouse writhing assay, a chemically evoked reflexive assay that measures pain intensity or severity as a function of the number of writhes elicited within the thirty(30) minute period following drug administration. Under this model we found that the control group, treated with normal saline, exhibited the highest number of writhes for each five (5) minute sub-period within this overall window. All other treatment groups showed increased analgesic response, as evidenced by a reduced number of writhes overall, with orphenadrine-diclofenac having the lowest count on average over all sub-periods, followed by diclofenac, and then orphenadrine, all of which nearly abolished writhing activity up until the last sub-period, indicative of analgesic activity.

Anxiety-like behaviour was assessed using the elevated plus maze, where both the number of entries and time spent in open and closed arms of the maze was recorded for all treatment groups. In the open arm, the control group saw the highest values for both the number of entries and time spent, with all other treatment groups showing far lower affinity for this arm of the maze, indicating increased avoidance of open space and suppressed exploratory drive across these groups that received active drug ($p < 0.01$, $p < 0.001$). The treatment group that received orphenadrine alone saw the lowest number of entries into this arm, compared to other groups, followed by the group that received diclofenac alone, which had a higher average value than the previously mentioned group, but not as high as that seen with the control group, and finally the treatment group that received the drug combination, indicating that analgesic administration overall, was associated with a moderate increase in exploratory and locomotor activity. All treatment groups saw similar values for the time spent in the closed arm, with only the control group

showing a slight decrease on average compared to all other groups. Now, anxiety-like behaviour was found to increase overall with drug treatment, as evidenced by the reduced number of entries and time spent in the open arms of the EPM, compared to the closed arm, across all treatment groups receiving active drugs. Seeing as analgesic administration led to increased avoidance behaviour and lowered exploratory and locomotor activity, we can infer that the drugs under study failed to down-regulate anxiety and consequent affective state, and instead exerted anxiogenic activity (Rodgers, 1997).

In view of quantifying the degree of oxidative stress in the CNS, malondialdehyde (MDA) levels in brain tissue were measured across all treatment groups, and from the results we can see that drug administration overall was associated with at least a five-fold increase in MDA levels compared to the control group ($p < 0.0001$), with the treatment group that received the drug combination having the lowest value, while the group that received diclofenac alone having the highest recorded value, indicating markedly high levels of oxidative damage, alongside the treatment group that received orphenadrine alone, which saw only a slight difference from the previously stated maximum. Malondialdehyde (MDA) is a by-product of lipid peroxidation and its levels in vivo are used to directly quantify the degree of oxidative stress. A number of studies have established a link between oxidative stress and the severity of acute pain, with elevated pain intensity being correlated with elevated lipid peroxidation and consequent MDA levels (Fischer et al, 2013). In this study however, this linear relationship was not exactly reflected, with the control group having the lowest levels of malondialdehyde, and other treatment groups, exhibiting greater levels of oxidative stress compared to the control, despite analgesic treatment. This is reflective of the fact that a positive correlation between pain and oxidative damage only exists in certain phenotypes of pain and is highly dependent on the nature and site of the underlying pathology (Fischer et al, 2013). Also, seeing as oxidative stress has been established to be positively correlated with anxiety-states and expression of anxiety-like behaviour,

elevated expression of MDA levels found across treatment groups to which analgesic agents were administered, was expected, as these groups also showed increased expression of anxiety-like behaviour. This increase in the degree of oxidative stress, may likely be linked and even causative of the anxiogenic activity of the drugs under study, as oxidative damage to neural structures is known to cause dysregulation of affective state and consequent behaviour, leading to conditions such as anxiety and depression (Smaga et al, 2014).

Peculiarities were found in the outcomes of the experiment, especially as regards the action of the orphenadrine-diclofenac combination. Administration of the drug combination was associated with decreased lipid peroxidation in the biochemical assay, and also an increased number of open arm entries in the behavioural assay compared to all other groups that received active analgesics. From these results, we can infer that the combination, at the doses administered, was associated with a slight reduction in the degree of oxidative stress and anxiogenic activity compared to other analgesic treatments. This outcome can be attributed to the combined mechanistic activity of the combination, abolishing peripheral inflammation, while simultaneously acting on pain processing via central neural pathways. It was also found that treatment groups receiving single analgesics reduced the number of closed arm entries compared to either the control group or the treatment group that received the drug combination. This effect was especially profound in the treatment group that received orphenadrine, which led to the observed increase in exploratory drive and locomotor activity, which is expected, as orphenadrine, a skeletal muscle relaxant, only has duration of action of 4-6 hours, after which paradoxical refractory effects such as anxiety may occur (Medicis Pharmaceutical Corporation, 2012).

While this study provides intriguing results, several limitations must be recognised that may limit its external validity and highlight the need for further research. For example, the EPM data, particularly for the orphenadrine group, is confounded by a likely decrease in overall locomotor activity, as evidenced

by reduced entries into both arm types, making it difficult to distinguish anxiogenic effects from sedation. The use of a single model for anxiety (EPM) and pain (writhing test) and a single biomarker, MDA, may only provide a partial picture of the overall activity profile of the treatments administered. Consequently, further research is essential and should employ a broader range of assays, such as unconditioned behavioral assays (like the Hole Board experiment), to distinguish anxiety from locomotor effects, and utilize a more markers of oxidative stress such as antioxidant enzymes like Catalase (CAT) and Glutathione (GSH), and also investigate dose-response relationships for individual treatments to clarify paradoxical effects and assess maximally effective dosage.

CHAPTER 5

5.0. CONCLUSION

This study sought to evaluate the behavioural and anxiotropic effects of orphenadrine, diclofenac, and their combination in an acute pain model in mice. The outcomes revealed a complex profile of activity across the analgesic, behavioural, and biochemical domains, leading to the following conclusions:

1. **Analgesic Efficacy**: All administered treatments successfully demonstrated analgesic activity in the acetic-acid induced writhing assay, as evidenced by a reduced number of writhes. The combination of orphenadrine and diclofenac was determined to be the most potent analgesic, having the lowest writhing count on average, followed by diclofenac alone, and then orphenadrine alone.
2. **Anxiogenic Activity**: Overall, drug treatments failed to down-regulate anxiety and behavioural state. Instead, analgesic administration led to increased avoidance behaviour and lowered exploratory and locomotor activity, indicating that the drugs under study exerted anxiogenic activity.
3. **Oxidative Damage**: Drug administration overall was associated with markedly high levels of oxidative damage, showing at least a five-fold increase in brain malondialdehyde (MDA) levels compared to the control group. This increase in the degree of oxidative stress is likely linked and even causative of the anxiogenic activity of the drugs under study.
4. **Combination Benefit**: The orphenadrine-diclofenac combination was associated with a slight reduction in the degree of oxidative stress and also exhibited limited anxiogenic activity compared to the single analgesic treatments. This beneficial outcome may be attributed to the combined mechanistic activity of the agents.

5.1. CONTRIBUTIONS TO KNOWLEDGE

This project work offers significant insight to the current understanding of the pathophysiology and pharmacotherapy of acute pain. The findings from the study provide preclinical evidence that two common analgesics, orphenadrine and diclofenac, may be associated with a paradoxical anxiogenic profile when administered in management of acute pain. This observation is quite significant as it suggests that pharmacological relief of pain does not automatically equate to an improvement in affective state. Furthermore, a strong correlation was established between the administration of these agents and a measurable increase in central oxidative stress (lipid peroxidation), offering a potential mechanistic pathway—drug-associated oxidative damage to neural structures—for the observed negative behavioural responses and reinforcing the established relationship between oxidative stress and dysregulation of affective state. The study also depicted a synergistic effect between orphenadrine and diclofenac, suggesting that the combination may offer a modest benefit in terms of reducing the high levels of oxidative stress observed with the single drug treatments.

5.2. RECOMMENDATIONS

Based on the findings of this study, future research should prioritise the isolation of the observed behavioural and biochemical effects, and also employ a broad range of behavioural assays, to distinguish between true expression of anxiety and general CNS modulation.

Further investigations should aim to fully characterize the biochemical mechanism underlying the increase in oxidative stress. This necessitates moving beyond the single biomarker (MDA) used here to incorporate a comprehensive panel of redox markers, including the activity of critical antioxidant enzymes like Glutathione (GSH) and Catalase (CAT), to gain a deeper understanding of the drug-induced oxidative imbalance. Additionally, research should seek to evaluate the external validity of these findings by testing the drug and combination effects across different pain phenotypes, such as thermal hyperalgesia or neuropathic pain models.

To translate these findings into clinical application, subsequent research must explore viable strategies to counteract the identified central side effects. This involves investigating the efficacy of co-administering these analgesics with known neuroprotective antioxidants to determine whether the negative affective and oxidative consequences can be prevented without compromising the level of pain relief. Ultimately, this work should inform the development of safer, next-generation analgesic regimens that are engineered not only for effective peripheral and central pain relief, but also for the preservation of CNS integrity and affective well-being.

REFERENCES

Bouayed, J, Rammal, H & Soulimani, R 2009, "Oxidative stress and anxiety: relationship and cellular pathways,"*Oxidative Medicine and Cellular Longevity*, 2(2):63–67,

Bouwknecht, JA & Paylor, R 2002, "Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains," *Behavioural Brain Research*, 136(2):489–501,

Chi, C-H, Shiesh, S-C & Lin, X-Z 2002, "Total antioxidant capacity and malondialdehyde in acute abdominal pain," The *American Journal of Emergency Medicine*, 20(2):79–82,

Cimpean, A & David, D 2019, "The mechanisms of pain tolerance and pain-related anxiety in acute pain," *Health Psychology Open*, 6(2)

Cocea, A-C & Stoica, CI 2024, "Interactions and Trends of Interleukins, PAI-1, CRP, and TNF-α in Inflammatory Responses during the Perioperative Period of Joint Arthroplasty: Implications for Pain Management—A Narrative Review," *Journal of Personalized Medicine*, 14(5):537,

Costigan, M & Woolf, CJ 2000, "Pain: Molecular mechanisms," *Journal of Pain*, 1(3):35–44, https://doi.org/10.1054/jpai.2000.9818.

Cryan, JF & Sweeney, FF 2011b, "The age of anxiety: role of animal models of anxiolytic action in drug discovery," *British Journal of Pharmacology*, 164(4):1129–1161,

De C Williams, AC 2023, "Pain," *Evolution Medicine and Public Health*, 11(1):429–437,

Del Rio, D, Stewart, AJ & Pellegrini, N 2005, "A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress," *Nutrition Metabolism and Cardiovascular Diseases*, 15(4):316–328, https://doi.org/10.1016/j.numecd.2005.05.003.

Fink, WA 2005, "The pathophysiology of acute Pain," *Emergency Medicine Clinics of North America*, 23(2):277–284,

Fischer, S, Perez, R, Nouta, J, Zuurmond, W & Scheffer, P 2013b, "Oxidative stress in complex regional pain syndrome (CRPS): No systemically elevated levels of malondialdehyde, F2-

Isoprostanes and 8OHDG in a selected sample of patients," *International Journal of Molecular Sciences*, 14(4):7784– 7794,

Fındıklı, E, Camkurt, MA, İzci, F, Karaaslan, MF, Fındıklı, HA, Sümer, P & Kurutaş, EB 2018d, "The diagnostic value of malondialdehyde, superoxide dismutase and catalase activity in drug naïve, First episode, Non-Smoker generalized Anxiety Disorder patients," *Clinical Psychopharmacology and Neuroscience*, 16(1):88–94,

Frijhoff, J, Winyard, PG, Zarkovic, N, Davies, SS, Stocker, R, Cheng, D, Knight, AR, Taylor, EL, Oettrich, J, Ruskovska, T, Gasparovic, AC, Cuadrado, A, Weber, D, Poulsen, HE, Grune, T, Schmidt, H HHW & Ghezzi, P 2015, "Clinical relevance of biomarkers of oxidative stress," Antioxidants *and Redox Signaling*, 23(14):1144–1170,

Haller, J & Alicki, M 2011, "Current animal models of anxiety, anxiety disorders, and anxiolytic drugs," *Current Opinion in Psychiatry*, 25(1):59–64,

Hånell, A. & Marklund, N 2014, "Structured evaluation of rodent behavioral tests used in drug discovery research", *Frontiers in Behavioral Neuroscience*, 8,

Harro, J 2017, "Animals, anxiety, and anxiety disorders: How to measure anxiety in rodents and why*Behavioural Brain Research*, 35281–93,

Hernández, V, Miranda, M, Pascual, I, Sánchiz, V, Almela, P, Añón, R, Cuadrado, E, Sanz, MI, Mínguez, M, Mora, F, Romero, FJ & Benages, A 2011b, "Malondialdehyde in early phase of acute pancreatitis," Revista *Española De Enfermedades Digestivas*, 103(11):563–569,

Ho, E, Galougahi, KK, Liu, C-C, Bhindi, R & Figtree, GA 2013, "Biological markers of oxidative stress: Applications to cardiovascular research and practice," *Redox Biology*, 1(1):483–491,

Hunskaar, S & Donnell, D 1991, "Clinical and Pharmacological Review of the Efficacy of Orphenadrine and Its Combination with Paracetamol in Painful Conditions," *Journal of International Medical Research*, 19(2):71–87,

Hunskaar, S, Berge, O-G & Hole, K 1985, "Antinociceptive effects of orphenadrine citrate in mice," *European Journal of Pharmacology*, 111(2):221–226,

Inanır, A, Sogut, E, Ayan, M & Inanır, S 2013, "Evaluation of Pain Intensity and Oxidative Stress Levels in Patients with Inflammatory and Non-Inflammatory Back Pain," *Electronic Journal of General Medicine*, 10(4):185–190,

Kornhuber, J, Parsons, CG, Hartmann, S, Retz, W, Kamolz, S, Thome, J & Riederer, P 1995, "Orphenadrine is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist: binding and patch clamp studies," *Journal of Neural Transmission*, 102(3):237–246,

Labout, J.J.M. *et al.* (1982) 'Difference between single and multiple dose pharmacokinetics of orphenadrine hydrochloride in man,' *European Journal of Clinical Pharmacology*, 21(4), pp. 343– 350.

Larson, CM, Wilcox, GL & Fairbanks, CA 2019, "The study of pain in rats and mice," *Comparative Medicine*, 69(6):555–570,

Lee, GI & Neumeister, MW 2020, "Pain," *Clinics in Plastic Surgery*, 47(2):173–180,

Lezak, KR, Missig, G & Carlezon, WA, Jr 2017, "Behavioral methods to study anxiety in rodents," *Dialogues in Clinical Neuroscience*, 19(2):181–191,

Matsumoto, K, Yobimoto, K, Huong, NTT, Abdel-Fattah, M, Van Hien, T & Watanabe, H 1999, "Psychological stress-induced enhancement of brain lipid peroxidation via nitric oxide systems and its modulation by anxiolytic and anxiogenic drugs in mice," *Brain Research*, 839(1):74–84,

McQuay, H 2009, "Acute pain management", *Cambridge University Press*,

Michaelides, A & Zis, P 2019c, "Depression, anxiety and acute pain: links and management challenges," *Postgraduate Medicine*, 131(7):438–444,

Mogil, JS 2009, "Animal models of pain: progress and challenges," *Nature Reviews. Neuroscience*, 10(4):283–294,

NORFLEX® (2012). *Orphenadrine citrate*. *Medicis Pharmaceutical Corporation*. Available at:

https://pdf.hres.ca/dpd_pm/00015942.PDF [Accessed 5 November 2025].

Ocktariyana, O, Lestari, D, Kadir, A, Listrianah, L & Handayani, H 2023, "The correlation between plasma malondialdehyde levels and pain in adolescent females diagnosed with primary dysmenorrhea," *Acta Biochimica Indonesiana*, 6(1):119,

Parent, AJ, Beaudet, N, Beaudry, H, Bergeron, J, Bérubé, P, Drolet, G, Sarret, P & Gendron, L 2012, "Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain," *Behavioural Brain Research*, 229(1):160–167,

Patel, PK, Sahu, J & Chandel, SS 2017, "A detailed review on nociceptive models for the screening of analgesic activity in experimental animals," *International Journal of Neurologic Physical Therapy*, 2(6):44,

Raja, SN, Carr, DB, Cohen, M, Finnerup, NB, Flor, H, Gibson, S, Keefe, FJ, Mogil, JS, Ringkamp, M, Sluka, KA, Song, X-J, Stevens, B, Sullivan, MD, Tutelman, PR, Ushida, T & Vader, K 2020, "The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises," *Pain*, 161(9):1976–1982,

Refsgaard, LK, Hoffmann-Petersen, J, Sahlholt, M, Pickering, DS & Andreasen, JT 2016, "Modelling affective pain in mice: Effects of inflammatory hypersensitivity on place escape/avoidance behaviour, anxiety and hedonic state," *Journal of Neuroscience Methods*, 26285–92,

Rodgers, RJ, Cao, B -j., Dalvi, A & Holmes, A 1997, "Animal models of anxiety: an ethological perspective," Brazilian *Journal of Medical and Biological Research*, 30(3):289–304,

Rodriguez, L 2015, "Pathophysiology of Pain: Implications for perioperative nursing," *AORN Journal*, 101(3):338–344,

Rosso, M, Wirz, R, Loretan, AV, Sutter, NA, Da Cunha, CTP, Jaric, I, Würbel, H & Voelkl, B 2022, "Reliability of common mouse behavioural tests of anxiety: A systematic review and meta-analysis on the effects of anxiolytics," *Neuroscience & Biobehavioral Reviews*, 143104928,

Singh, L, Bilwal, G, Godara D 2019, "Evaluation of various techniques to determine in analgesic activity," *World Journal of Pharmaceutical Research*, 8(3):429–439

Smaga, I, Niedzielska, E, Gawlik, M, Moniczewski, A, Krzek, J, Przegaliński, E, Pera, J & Filip, M 2015, "Oxidative stress as an etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism," *Pharmacological Reports*, 67(3):569–580,

Smith, HS, Pappagallo, M & Stahl, SM 2012, "Orphenadrine," in *Cambridge University Press eBooks*, pp. 364–366,

Tejchman, K, Kotfis, K & Sieńko, J 2021, "Biomarkers and Mechanisms of Oxidative Stress—Last 20 Years of Research with an Emphasis on Kidney Damage and Renal Transplantation," *International Journal of Molecular Sciences*, 22(15):8010,

Vujovic, KS, Zivkovic, A, Dozic, I, Cirkovic, A, Medic, B, Srebro, D, Vuckovic, S, Milovanovic, J & Jotic, A 2023, "Oxidative Stress and Inflammation Biomarkers in Postoperative Pain Modulation in Surgically Treated Patients with Laryngeal Cancer—Pilot Study," *Cells*, 12(10):1391,

Walf, A. A.& Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behaviour in rodents. *Nature Protocols*, 2(2), 322-328.

Whittaker, AL & Howarth, GS 2013, "Use of spontaneous behaviour measures to assess pain in laboratory rats and mice: How are we progressing?," *Applied Animal Behaviour Science*, 1511–12,

Xiao, X, Ding, M & Zhang, Y-Q 2021, "Role of the anterior cingulate cortex in translational pain research," *Neuroscience Bulletin*, 37(3):405–422,

Zhang, Q, Sun, H, Xin, Y, Li, X, Shao, Do 2024, "Studies on Pain Associated with Anxiety or Depression in the Last 10 Years: A Bibliometric Analysis," *Journal of Pain Research*, 17: 133-149

APPENDIX

The dose administered was calculated using the formula:

$$Dose\ to\ administer\ (mL)\ =\ \frac{Weight\ of\ the\ animal\ (kg)\ \times Dose\ (mg/kg)}{Concentration\ of\ the\ stock\ solution\ (mg/mL)}$$

Table 2.1: Body Weight and Volume of Normal Saline (10ml/kg) administered to the first treatment group (control group, marked green)

Number of marks on tail	Weight (g)	Volume of Normal Saline (mL)
I	22.8	0.23
II	21.3	0.21
III	22.1	0.22
IV	22.1	0.22
V	23.0	0.23
(Head Marked)	20.8	0.21`

Table 2.2: Body Weight and Volume of Orphenadrine (25mg/kg, 1mg/mL) administered to the second treatment group (marked blue)

Number of marks on tail	Weight (g)	Volume of orphenadrine stock solution (mL)
I	20.3	0.50
II	22.1	0.55
III	22.2	0.56
IV	22.6	0.57
V	21.5	0.54

(Head Marked)	21.1	0.53

Table 2.3: Body Weight and Volume of Diclofenac (50mg/kg, 2.5mg/mL) administered to the third treatment group (marked red)

Number of marks on tail	Weight (g)	Volume of Diclofenac stock solution (mL)
I	26.4	0.53
II	24.1	0.48
III	26.0	0.52
IV	28.6	0.57
V	29.4	0.59
(Head Marked)	26.4	0.53

Table2.4: Body Weight and Volume of Orphenadrine (25mg/kg, 1mg/kg) and Diclofenac (50mg/kg, 2.5mg/mL) administered to the fourth treatment group (marked black)

Number of marks on tail	Weight (g)	Volume of Orphenadrine Stock solution (mL)	Volume of Diclofenac stock solution (mL)
I	25.2	0.63	0.50
II	24.6	0.62	0.49
III	23.8	0.60	0.48
IV	23.3	0.58	0.47
V	25.3	0.63	0.51
(Head Marked)	25.4	0.64	0.51