

**PHYSIOLOGICAL RESPONSES OF WISTAR RATS TO EXPERIMENTALLY-
INDUCED PAIN DURING THE HARMATTAN SEASON AND THE
MODULATORY ROLE OF RESVERATROL**

By

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Declaration

I declare that the work in this thesis, entitled ‘Physiological responses of Wistar rats to experimentally-induced pain during the harmattan season and the modulatory role of resveratrol’ has been carried out by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Professor J. O. Ayo. Dr R. A. Magaji and Dr M. G. Magaji.

The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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This thesis entitled “PHYSIOLOGICAL RESPONSES OF WISTAR RATS TO EXPERIMENTALLY-INDUCED PAIN DURING THE HARMATTAN SEASON AND THE MODULATORY ROLE OF RESVERATROL” by Ahmed-Sherif ISA meets the regulations governing the award of the degree of Doctor of Philosophy in Human physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This thesis is dedicated to my parents Col. O.A.U. (rtd.) and Mallama Hafsatu Sadiku

and

To all physiologists aspiring against all odds to be relevant in the field of scientific research

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Abstract

This study investigated the influence of harmattan season (November-February) in naïve animals subjected to mechanical, chemical and thermal noxious stimulation and the modulating effect of resveratrol. Animals were grouped into two major groups of 50 males and females respectively. These major groups were subdivided into control, carboxymethyl cellulose (CMC), resveratrol (RSV) groups with 5 animals each. Different sets of animals were subjected separately to mechanical, chemical and thermal noxious stimulation. Dry bulb temperature readings were taken at 06:00 h, 13:00 h and 18:00 h during the course of the study. After determination of pain thresholds for the three different noxious stimuli, animals were sacrificed and serum was collected for biochemical analyses of sodium, potassium and chloride concentration. Determination of malondialdehyde, superoxide dismutase concentration and erythrocyte osmotic fragility were also carried out. The results obtained revealed that responses to mechanical, chemical and thermal noxious stimuli did not show significantly variation between male and female animals even though females had higher pain sensitivity. Mechanical pain threshold was higher in the morning (06:00 h) and lowest in the evening (18:00 h). Thermal pain threshold was positively correlated ($p < 0.05$) with time. Resveratrol showed no significant analgesic activity in all the experimentally induced pain paradigms, but CMC groups had significantly lower thresholds when compared to other groups. Sodium ion concentration was highest in animals exposed to mechanical noxious stimulation and lowest in animals exposed to thermal noxious stimulation. Sodium and potassium ion concentrations was significantly higher in male than female animals. Percentage haemolysis of erythrocyte was significantly higher ($p < 0.05$) in animals subjected to mechanical and chemical noxious stimulation when compared to animals subjected to thermal noxious stimulation. This results were further corroborated by increased

malondialdehyde concentration and superoxide dismutase activity in animals subjected to mechanical and chemical noxious stimulation. The finding of this study suggests that diurnal variation in pain responses to various noxious stimuli during the harmattan is similar in male and female rats. Also resveratrol did not possess any analgesic properties during the harmattan season. Lastly, mechanical noxious stimulus produced the highest oxidative damage than the chemical and thermal noxious stimulation.

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Abbreviations

| | |
|-----------------------------------|---|
| ACC | Anterior cingulate cortex |
| AMPA | α -amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | Analysis of variance |
| ASIC | Acid-sensitive ion channels |
| ATP | Adenosine triphosphate |
| Ca²⁺ | Calcium ion |
| CMC | Carboxymethyl cellulose |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase-2 |
| CREB | cAMP Response Element-Binding Protein |
| CRTC1 | CREB-regulated transcription co-activator 1 |
| DEG/ENAC | Degenerin /epithelial sodium channels |
| DRG | Dorsal root ganglia |
| EDTA | Ethylenediaminetetraacetic acid |
| ENaCs | Epithelial/degenerin Na ⁺ channels |
| H⁺ | Hydrogen ion |
| H₂O₂ | Hydrogen peroxide |
| HO[·] | Hydroxyl radical |
| HO-1 | Haemeoxygenase1 |
| HVA | High-voltage-activated |
| IASP | International Association for the Study of Pain |
| K⁺ | Potassium ion |
| LVA | Low-voltage activated |

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|-----------------------------------|--|
| MDA | Malondialdehyde |
| MnSOD | Mitochondrial superoxide dismutase |
| Na⁺ | Sodium ion |
| NaCl | Sodium chloride |
| NADA | N-arachidonyl dopamine |
| NMDA | N-Methyl-D-aspartate |
| NMDAR | N-Methyl-D-aspartate receptor |
| NO | Nitric oxide |
| Nrf2 | Nuclear factor E2-related factor 2 |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| O₂^{·-} | Superoxide |
| ONOO⁻ | Peroxynitrite |
| PAG | Periaqueductal grey |
| RMN | Raphe magnus nucleus |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RVM | Rostro-ventral medulla |
| SIRT1 | Silent Information Regulator T1 |
| STT | Spinothalamic tract |
| TBA | Thiobarbituric acid |
| TG | Trigeminal ganglion |
| TRP | Transient receptor potential |
| TRPA | Transient receptor potential (Ankyrin) |
| TRPC | Transient receptor potential (Canonical) |

| | |
|-------------|---|
| TRPM | Transient receptor potential (Melastatin) |
| TRPV | Transient receptor potential (Vanilloid) |
| TTX | Tetrodotoxin |
| UV | Ultraviolet |
| VR | Vanilloid receptor |

CHAPTER ONE

1.1 Introduction

The word pain is thought to be derived from the Latin word *poena*, meaning punishment (Gu *et al.*, 2005). “Pain is an unpleasant sensory and emotional experience, associated with actual or potential tissue damage, or described in terms of such damage” (Merskey, 1979). Pain is a widespread clinical problem that imposes significant financial burdens due to long-term treatment (Bhangoo and Swanson, 2012). Pain is a common and distressing symptom encountered by individuals, which limits efficiency and diminishes quality of life (Caraceni *et al.*, 2002; Mert *et al.*, 2013).

Pain triggers various responses in the spinal cord and the brain, including reflexes, conscious perception, cognitive learning and memory processes, emotional reactions such as depression, and drug addiction (Gu *et al.*, 2005). Pain is a warning signal of tissue damage, resulting from an accidental trauma, infection, or inflammation. Indeed, it is one of the cardinal signs of inflammation that usually disappears after the injured tissue has healed (Sessle, 2012). Pain is considered as a primary physiologic defence mechanism, which protects the body against infection, burns, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many chronic illnesses (Singh *et al.*, 2009).

Pain sensation can be influenced by many factors, which include the strength of the noxious stimulus, state of the organism, and environmental variables (Strigo *et al.*, 2000). Environmental temperature may be one important variable in nociceptive processing. The relationship between changes in weather and pain perception has been documented since the

classical Roman age. Hippocrates was the first to note, in about 400 B.C. that many illnesses were related to changes in season (Jamison, 1996). The various meteorological factors that have been suspected of contributing to changes in pain include ambient temperature, barometric pressure, precipitation, humidity, thunderstorms, sunshine, and increased ionisation of the air (Sulman *et al.*, 1984; Harlfinger 1991). Even seasonal changes in testosterone and cortisol levels in the body have been shown to influence pain responses (Choi *et al.*, 2014). Certain pain conditions have been reported to be especially sensitive to weather changes, especially rheumatoid arthritis (Jamison, 1996). Weather exerts influence on health and disease in humans and animals (Gerber *et al.*, 2006; Honda *et al.*, 2016; Jones and Mays, 2016). The response of man and animal to noxious stimuli may vary based on the microclimatic parameters surrounding man in work places, especially during the various seasons of the year and in a tropical environment. Extremely low or high environmental temperatures are reported to produce opioid-mediated stress analgesia (Strigo *et al.*, 2000).

Accumulating evidence has implicated oxidative stress in many diseases, especially in diabetes mellitus, atherosclerosis, neurodegenerative diseases, cancer and ageing (Pazdro and Burgess, 2012). Pain has also been reported to increase the generation of reactive oxygen species (ROS) and the administration of antioxidants in pain decreases the doses of analgesics (Rokyta *et al.*, 2003; Chauhan *et al.*, 2012; Salat *et al.*, 2013).

Resveratrol is a polyphenolic compound, found in grapes and peanut. It possesses multiple pharmacological actions, exerting mainly anti-inflammatory, anti-tumour, immunomodulatory, neuroprotective and potent antioxidant effects (Candelario-Jalil *et al.*, 2007; Harb *et al.*, 2009; Cenesiz *et al.*, 2012). Resveratrol is effective in models of acute

(hot-plate), inflammatory (formalin), carrageenan, neuropathic (spinal nerve ligation) and diabetic pains (Montiel-Ruiz, 2011). Studies have also shown that resveratrol attenuates thermal hyperalgesia and cold allodynia in diabetic-induced neuropathy (Sharma *et al.*, 2006).

The molecular mechanism of resveratrol activity on pain is associated with the inhibition of Na⁺ currents in dorsal root ganglion and the activation of K⁺ channels. Furthermore, the antinociceptive effect of resveratrol has been related to the inhibition of activity or expression of cyclooxygenase-2 (COX-2), reduction of oxidative stress, and down-regulation of pro-inflammatory cytokines (Montiel-Ruiz, 2011). Potassium channels have been implicated in pain perception and the opening of potassium channels have been demonstrated to confer antinociceptive effects or responses. Non-steroidal anti-inflammatory drugs (NSAIDs) and resveratrol have been reported to relieve hyperalgesia through opening of potassium channels (Granados-Soto *et al.*, 2002).

The Northern Guinea Savannah zone of Nigeria is characterized by three different seasons: hot-dry, hot-humid and harmattan (Igono and Aliu, 1982). The hot-dry season (traditionally, the dry season), which lasts between March and April is characterized by low precipitation, very high ambient temperature maxima (35.5 ± 0.8 °C), low relative humidity ($27.5 \pm 6.1\%$) and extremely high evaporation rate. The hot-humid (rainy) season (May-October) has very high rainfall, high mean ambient temperature maxima (30.2 ± 0.6 °C), very high mean relative humidity ($63.2 \pm 5.6\%$) and low evaporative rate. The harmattan season (November to February) has no rains, but characterized by low ambient temperature minima (14.7 ± 1.0

°C), low relative humidity ($16.9 \pm 2.4\%$) and moderate evaporation rate (Ayo *et al.*, 2011; Dzenda *et al.*, 2011).

The relationship between pain response and meteorological changes has been reported, though only a few studies have investigated this relationship, and many questions remain unanswered. The current study intends to investigate the physiological responses of naïve Wistar rats (as an animal model) to pain, including pain-induced biochemical changes in the rats during the harmattan season (November-February), in the Northern Guinea Savannah zone of Nigeria.

1.2 Statement of Research Problem

Studies have suggested that perception of pain may be highly variable across individuals and throughout the circadian cycle (Odrich *et al.*, 2006). These may suggest that environmental temperatures alter pain perception in humans and animals. However, the data obtained from previous investigations are inconsistent and the existing paucity of information remains to be filled.

Cool temperatures have been reported to produce antinociception by increasing the response latency of rats in hot plate and tail flick models of pain (Strigo *et al.*, 2000). However, the effect of warm climate is controversial, because both analgesic and hyperalgesic effects have been reported (Deeter and Mueller, 1981; Schoenfeld *et al.*, 1985) or fluctuations of high and low temperatures that characterize the harmattan season (November-February).

Extensive reports on sex differences in pain have been written in recent years, although differences in males and females have been reported, these reports are not always consistent, in general it seems the female sex are predisposed to higher pain sensitivity and risk to clinical pain (Bartley and Fillingim, 2013). The present study attempts to investigate the effect of the harmattan season on pain sensitivity of male and female Wistar rats to noxious mechanical, thermal and chemical stimuli, and the modulatory effect of resveratrol administration.

1.3 Justification

Seasonal and diurnal variation of various physiological variables have been reported. They include cortisol, opioids, salivary hormones, melatonin etc. More importantly, pain disorders syndromes or symptoms have also been described to be influenced by weather, seasons or circadian variation some of disease conditions include most notably rheumatism, osteoarthritis, migraine (Bellamy *et al.*, 2002; Hoffman *et al.*, 2011; Brennan *et al.*, 2012). However, there are few reports on these seasonal variations in these pain syndromes and fewer reports on diurnal and seasonal changes in physiological aspects of pain in naïve organisms. It is important to note that seasonal and diurnal variations are not only observed in animals in the natural environment but can also be obtained in standard laboratory conditions (Nagayama and Liu, 1998).

Investigating seasonal and diurnal fluctuations in pain responses in naïve animals will deepen our knowledge in pain mechanisms and provide clues for further understanding of this phenomenon.

1.4 General Aim

The aim of this study was to investigate the physiological responses of rats to mechanical, chemical and thermal noxious stimuli during the harmattan season (November-February), and the modulatory effects of resveratrol.

1.5 Specific Objectives

The specific objectives of this study were to:

- i. Evaluate mechanical pain sensitivity in male and female Wistar rats during the harmattan season (November-February), using the Randall-Selitto model and the modulatory role of resveratrol.
- ii. Evaluate thermal pain sensitivity in male and female Wistar rats during the harmattan season (November-February), using the hot-plate model and the modulatory role of resveratrol.
- iii. Evaluate chemical pain sensitivity in male and female Wistar rats during the harmattan season (November-February), using the formalin induced pain test and the modulatory role of resveratrol.
- iv. Evaluate the biochemical changes induced by pain responses to mechanical, chemical and thermal noxious stimuli in male and female Wistar rats during the harmattan season (November-February), using the assessment of oxidative stress parameters (malondialdehyde, superoxide dismutase), erythrocyte osmotic fragility and serum electrolyte concentration: sodium, potassium and chloride.

1.6 Hypothesis

Physiological and biochemical responses of rats to pain are not modified during the harmattan season and resveratrol may modulate these responses during the harmattan season.

CHAPTER TWO

2.0 Literature Review

2.1 Classification and Type of Pain

Pain can be classified based on several variables, including its duration (acute, convalescent, chronic), pathophysiologic mechanisms (physiologic, nociceptive, neuropathic), and clinical context for example, post-surgical, malignancy related, neuropathic, degenerative (Vadivelu *et al.*, 2009). Pain is an early warning system that serves a protective function, which helps to limit exposure to damaging noxious stimuli; thus, detection of noxious stimuli to prevent further contact is described as nociceptive pain (Woolf, 2010). When a noxious stimulus is capable of provoking real or potential injury, not necessarily causing injury, the pain experienced by virtue of this type of stimulus is referred to as nociceptive pain (Almeida *et al.*, 2004). It is a high-threshold pain initiated only in the presence of intense noxious stimuli. Nociceptive pain is not a clinical problem, except from the surgical perspective where nociceptive is suppressed with the use of anaesthetics. A second kind of pain is described as being adaptive and protective by increasing pain sensitivity (low threshold to pain) to avoid tissue damage, thus allowing for proper healing by limiting physical movement. This type of pain is called inflammatory pain because it involves the activation of the immune system by tissue injury (Woolf, 2010). The inflammation results in the generation of a plethora of chemical agents that are intended to fight infection and assist in the repair of injured tissue (White *et al.*, 2010). Unfortunately, the body's inflammatory response to injury, or disease, is often disproportionate, resulting in pain that is sometimes of such severity that it may adversely affect recovery, and in the longer term result in disability (White *et al.*, 2010).

The third type of pain is not adaptive, lacks any protective role and serves no useful purpose; it is referred to as pathological pain (Meintjes, 2012). It may result from abnormal functioning of the nervous system (neuropathic pain) or pain elicited in the absence of any noxious stimuli (dysfunctional pain). It is characterized by intensified pain sensitivity, low threshold of pain in the absence of any noxious stimulus. Pathological pain has clinical significance and accounts for patients seeking medical attention (Woolf, 2010). Based on aetiology and neurobiological mechanisms, the following, different types of pain can be distinguished: (i) nociceptive pain, caused by any lesion or potential tissue damage; (ii) inflammatory pain, due to inflammatory processes; and (iii) neuropathic pain, induced by a lesion or disease affecting the somatosensory system (Verdu *et al.*, 2008).

Classification of pain based on speed include fast/first pain (pin prick pain), which is a sharp localized and last no longer than the applied stimulus, usually accompanied by withdrawal reflex (Motoc *et al.*, 2010). Secondary/second pain is characterized as burning, aching, long lasting and mediated by inflammatory substances as a result of tissue damage (Motoc *et al.*, 2010; Meintjes, 2012). Pain can also be classified as physiologic and clinical pain (neuropathic and inflammatory pain) Clinical pain can arise either from damage to the nervous system (neuropathic pain) or inflammatory states (inflammatory pain) (Cury *et al.*, 2011).

Chronic pain can be described as pain persisting for more than 30 days, 3 months, 6 months or 12 months. Chronic pain is sometimes referred to as persistent pain. It could be described as pain that extends beyond the expected period of healing while acute pain is typically temporary; resulting from traumatic tissue injury or infection and it is generally limited in

duration (Vadivelu *et al.*, 2009; Meintjes, 2012). Chronic pain may have multiple causes and is characterized by gradual onset. It may be symptomatic or diagnostic and serves no adaptive purpose, and may be refractory to treatment. Acute pain usually results from obvious tissue damage, has distinct onset, well localized, resolves after healing, has a useful biologic purpose and responds effectively to treatment (Vadivelu *et al.*, 2009; Sweiboda *et al.*, 2013). A noxious stimulus is capable of provoking a real or potential injury, not necessarily causing pain. In this vein, pain experienced by virtue of this type of stimulus is characterized as nociceptive pain. However, it is known that the painful phenomenon can occur spontaneously, as is the case for non-nociceptive pain, represented by the reduction of the receptor thresholds due to alterations of the central nervous system. According to the International Association for the Study of Pain (IASP) definition, the relation between pain and degree of injury is not obligatory. Thus, the alert function applies only to an acute manifestation; that is, the one that follows damage to the tissue. Acute pain is characterized by delimitation in time and disappearing with the resolution of the pathological process. Chronic pain that persists for an extended period of time is associated with chronic pathological processes and causes suffering in multiple systems (Almeida, 2004).

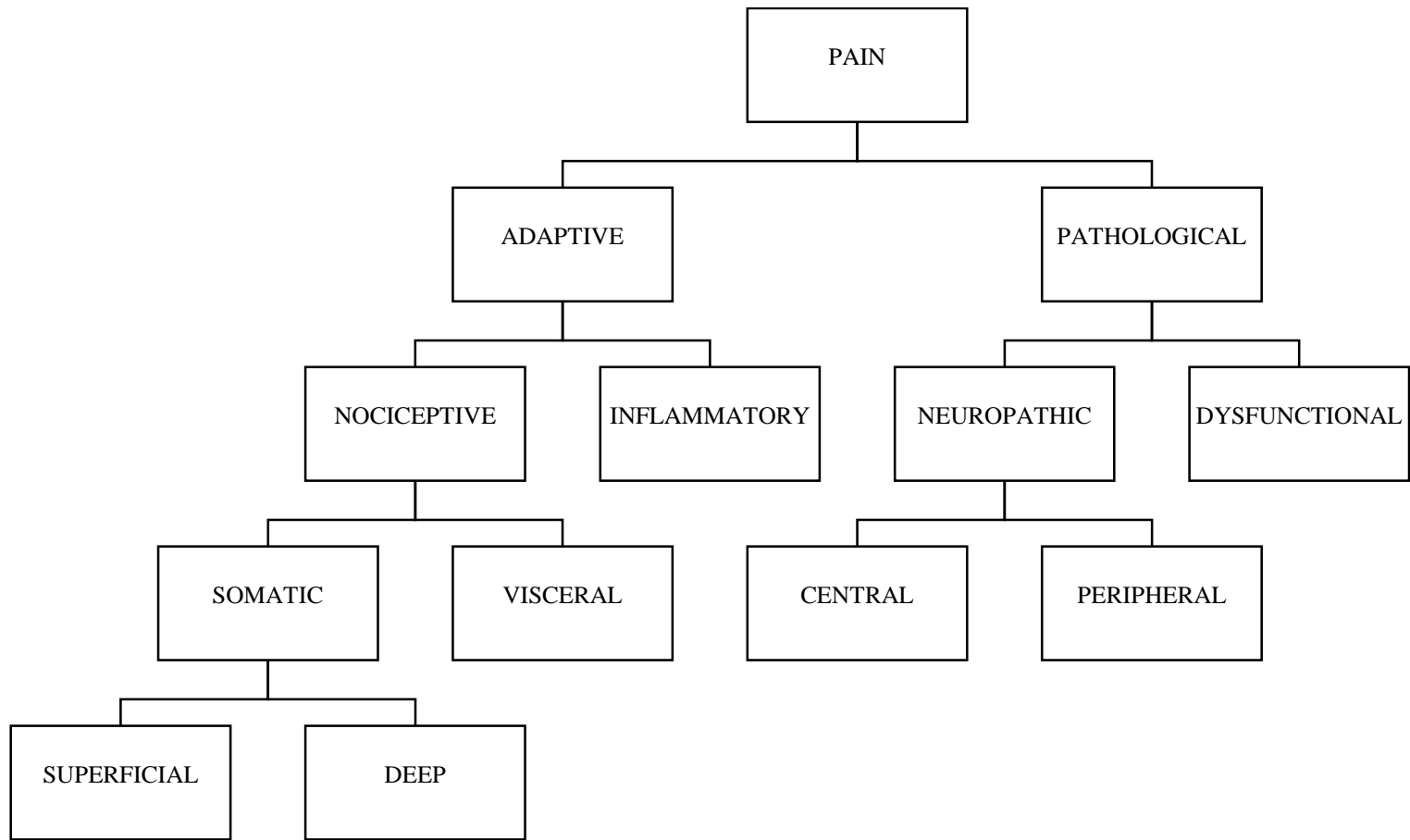


Figure 2.1: Classification of Pain (Woolf, 2010)

2.2 Significance of Pain

The necessity of an organism to sense potential harm in the environment is very important. Pain receptors help to detect noxious stimuli that often trigger pain sensations in order to promote avoidance and protect the organism from further harm (Riera *et al.*, 2014). Pain and its associated responses can be unpleasant and debilitating, but they serve important adaptive purposes. Pain serves as an essential process to localize noxious stimuli, initiate withdrawal responses that minimize tissue injury and limit movement, thereby enhancing/promoting wound healing. It also initiates and modifies future behavioural responses (Vadivelu *et al.*, 2006; Rahman and Dickenson, 2013).

Inability to perceive pain, as in the rare condition of congenital insensitivity to pain, can cause very serious health problems such as self-mutilation, auto-amputation, and corneal scarring (Fein, 2012). Insensitivity to pain due to loss of function, missense or mutation of SCN9A, the gene that codes for voltage-gated sodium channel Nav1.7. This gene could be harmful because it can lead to self-mutilation or excessive use of an osteoarthritic joint and loss of the protective role of pain (Woolf, 2010). In summary, pain is a self-preservative mechanism that occurs as the brain's protective response to electrical and chemical changes that appear as a result of injury, disease or damage to the body (Gohar, 2005).

2.3 Nociceptive Processing and Pathways

Perception of pain involves the activation of peripheral nociceptors, generation of nerve signal and the transmission of this signal to the somatosensory cortex (Merighi *et al.*, 2008). Conveyance of nociceptive stimuli follows different pathways, which consist at least three

neurones; (i) a first order sensory neurone in the dorsal root ganglia (DRGs); (ii) a second order neurone in the spinal cord dorsal horn; (iii) a third order neurone which is generally located in the ventral posterolateral nucleus of the thalamus (Merighi *et al.*, 2008).

A noxious stimulus applied to the body is initially detected by nociceptors on the peripheral axons of primary sensory neurones. These primary sensory neurones, whose cell bodies are located in the dorsal root ganglion (DRG) or trigeminal ganglion (TG), transmit nociceptive information to secondary neurones in the spinal or medullary dorsal horn. In the dorsal horn, the nociceptive and non-nociceptive information is integrated and processed by complex circuits involving excitatory and inhibitory interneurones and descending axons from the brainstem as well as glial cells. The processed information is then transmitted via the ascending pathway to multiple brain areas where pain sensation and associated negative effects and emotions are elicited. These brain areas include not only somatosensory cortices, which are mainly involved in the sensory aspect of pain, but also limbic systems and other cortices such as the amygdala, anterior cingulate cortex, insular cortex and prefrontal cortex. These structures are considered to be involved in the affective and emotional aspects of pain as well as pain perception (Sakai and Suzuki, 2014)

2.3.1 Peripheral receptors and sensory neurones

Nociception is the process by which intense thermal, mechanical, or chemical stimuli are detected and propagated by a subpopulation of peripheral nerve fibres, called nociceptors (Basbaum *et al.*, 2009). Nociceptors are widely distributed in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules,

periosteum, muscles, tendons, and arterial vessels (Stucky *et al.*, 2001; Gold and Gebhart, 2010). Nociceptors are specific and are high excitability threshold sensory neurones that respond only to noxious stimuli (Woolf, 2011). They are also peripheral receptors that represent the end segment of the unmyelinated and myelinated neurones (Motoc *et al.*, 2010). Nociceptors respond selectively to stimuli that cause damage or threaten to cause damage (Willis, 2007). They specifically respond to a type of noxious stimuli, such as noxious mechanical, thermal and chemical stimuli. Some other nociceptors are polymodal and they respond to more than one type of noxious stimuli (Willis, 2007). These receptors are free nervous endings, located in the distal part of the first-order afferent neurone which is made of either small-diameter fibres, with little or no myelinated, that corresponds to the A δ or C type, respectively (Almeida *et al.*, 2004).

First-order afferent fibres are classified in terms of structure, diameter, and conduction velocity (Millan, 1999; Almeida *et al.*, 2004). C-type fibres are unmyelinated, ranging in diameter from 0.4 to 1.2 μm and have a velocity of 0.5–2.0 m/s; A-Delta fibres are barely myelinated, ranging in diameter from 2.0 to 6.0 μm , and have a velocity of 12–30 m/s. The A-Beta fibres are myelinated, with a diameter of more than 10 μm and a velocity of 30–100 m/s, and do not propagate noxious potentials in normal situations. However, they are fundamental in the painful circuitry because they participate in the mechanisms of segmental suppression (Millan, 1999). The A-Delta fibres carry the sensation of fast pain, first pain or acute pain, while the C-type fibres carry the sensation of slow pain, second pain or chronic pain (Almeida *et al.*, 2004).

Under physiological conditions, the painful sensation results from specific activation of the nociceptors by mechanical, thermal, or chemical stimulus, and not by the hyperactivity of other sensory modality receptors. The threshold of these nociceptors are higher than the other receptors and they respond according to the intensity of the stimulus. Sensitisation of these nociceptors causes reduction of the thresholds and, in some cases, spontaneous activity (Millan, 1999; Almeida *et al.*, 2004). C fibres with high-threshold receptors are sensitive to mechanical, thermal and chemical stimuli, and for this reason are called polymodal (Almeida *et al.*, 2004).

Nociceptive (and non-nociceptive) primary afferent neurones are housed in the DRGs, and the sensory ganglia associated with certain cranial nerves (Merighi *et al.*, 2008). These neurones are referred to as pseudo-unipolar because they give rise to a single main process, splitting into a peripheral and a central branch (Basbaum *et al.*, 2009). The peripheral branch of DRG neurones terminates in different tissues and organs to receive different sensory modalities (Merighi *et al.*, 2008; Basbaum *et al.*, 2009), whereas the central branch transmits impulse sensations to the spinal cord via the dorsal horns (Merighi *et al.*, 2008).

Thin-myelinated fibres are likely to reach lamina V, while C fibres end in laminae I and II. Nociceptive PAFs originating from sensory neurones from the head and neck regions terminate within the trigeminal spinal nucleus (Merighi *et al.*, 2008). Unmyelinated, C afferent fibres, and myelinated A δ afferent fibres, which are responsive to noxious chemical, thermal and mechanical stimuli, convey nociceptive signals to superficial (laminae I/II) and

deep (V/VI) laminae of the dorsal horn (Millan, 2002). While myelinated, A β fibres convey innocuous, mechanical stimuli to deeper laminae (III–VI) (Millan, 2002).

2.3.2 Ascending Pathways

The ascending spinal pathways are responsible for the transmission of nociceptive stimulus to the higher centers. The spino-thalamic pathway is conventionally known to be the main nociceptive pathway (Merighi *et al.*, 2008).

2.3.2.1 Spinothalamic tract

The neurones of the Spinothalamic tract (STT) are localized in lamina I and in deeper laminae (IV–VI), especially at the base of the medial aspect of dorsal horn, ascend in the ventro-lateral funiculus and terminate in the lateral thalamus. Other neurones located at the base of the medial aspect of dorsal horn and in the ventral horn (laminae VII–VIII) ascend in the ventral funiculus and terminate in the medial thalamus. Some STT neurones located in lamina I and the lateral spinal nucleus may project to the medial thalamus through the dorso-lateral funiculus that is sometimes referred to as dorsal STT (Merighi *et al.*, 2008).

Collectively, the terminals of STT neurones terminate in either of the three main thalamic regions: (i) the ventrobasal complex; (ii) the medial thalamus including the intralaminar nuclei; and (iii) the posterior complex, an important processing site for pain integration (Salt *et al.*, 2014). The neurones in the ventrobasal complex are mostly responsible for sensory and discriminative aspects of noxious stimuli, while the neurones in the intralaminar

nuclei/posterior complex are responsible for motivational and emotional aspects of pain, as well as the escape reaction to acute pain (Merighi *et al.*, 2008).

The thalamus is an essential relay for the reception and processing of nociceptive information en route to the cortex. The thalamo-cortical inputs are mainly conveyed to the first somato-sensory area of the post-central cortical gyrus (S I) (Merighi *et al.*, 2008; Salt *et al.*, 2014). Although other cortical areas are reported to be activated by noxious stimuli, such as: the second somato-sensory area, certain regions of the parietal cortex, the insular cortex, the anterior cingulate cortex, and the medial prefrontal cortex (Lehner *et al.*, 2004). There are other ascending pathways associated with pain which are less well-characterized and less critical for sensory-discrimination of pain, notwithstanding they are also important for pain sensation and pain control (Merighi *et al.*, 2008). These other ascending pathways involved in pain conduction and transmission include: spino-reticular, spino-mesencephalic, spino-hypothalamic, spino-parabrachial tracts. These ascending pathways may participate in motivational, affective, cognitive and autonomic aspects of pain processing (Milan, 1999; Willis, 2007; Merighi *et al.*, 2008).

2.3.2.2 Spino-reticular tracts

Spino-reticular tracts originate from laminae I, V/VI, and X. They reach the lateral reticular nucleus or the medial nuclei of the ponto-medullary reticular formation. The spino-reticular tract is involved in the motivational and cognitive aspect of pain processing, as well as in the activation of the descending inhibition (Merighi *et al.*, 2008; Steeds, 2016).

2.3.2.3 Spino-mesencephalic tract

The spino-mesencephalic tract originates from neurones found in almost all dorsal horn laminae (Merighi *et al.*, 2008). Their fibres reach the periaqueductal grey (PAG), the superior colliculus and the parabrachial nucleus. This pathway is involved in motor responses to pain and in affective aspects (Milan, 1999).

2.3.2.4 Spino-hypothalamic tract

The spino-hypothalamic tract originates from neurones in laminae I, V and X. Axons reach the contralateral hypothalamus. This pathway is involved in neuro-endocrine/autonomic responses to pain stimuli (Merighi *et al.*, 2008).

2.3.2.5 Spino-parabrachial tract

The spino-parabrachial tract originates from nociceptive specific lamina I/II neurones sending their axons to the parabrachial nucleus, Third-order projection neurones of parabrachial nuclei send their axons to the amygdala, the bed nucleus of the stria terminalis and the hypothalamus. This pathway is mainly dedicated to the motivational affective representation of pain information (Merighi *et al.*, 2008; Braz *et al.*, 2014; Walker, 2014).

2.3.3 Descending pathways

The rostro-ventral medulla (RVM) is the major origin of descending pathways. It plays an important role in the modulation and integration of nociceptive information within the dorsal horn (Merighi *et al.*, 2008), but there are other brain regions involved in descending modulation of pain and they include the frontal lobe, anterior cingulate cortex (ACC), insula,

amygdala, hypothalamus, PAG and nucleus cuneiformis (Tracey and Mantyh, 2007). Compelling evidence has established that stimulation of RVM can inhibit and/or facilitate nociceptive processing; thus, most descending pathways exert an inhibitory control onto the excitability of dorsal horn neurones, although some facilitatory effects have also been reported (Porreca *et al.*, 2002).

2.3.3.1 Descending inhibitory pathway

The main inhibitory descending pathway is the raphe-spinal tract, which originates from the medullary raphe magnus nucleus (RMN). Other descending pathways include projections from the medullary reticular nuclei of the gigantocellular complex and the locus coeruleus and are confined to the dorsolateral funiculi (Gebhart, 2004; Merighi *et al.*, 2008). Inhibitory inputs originating from PAG as a result of removal of tonic inhibition of local enkephalinergic neurones, are relayed through the medulla; specifically, the RMN, including the medial nucleus raphe magnus (Gebhart, 2004). This inhibition of inhibitors is mediated by the intervention of hypothalamic afferents that release opioid peptides (β -endorphin) in response to specific sensory inputs from ascending pathways.

Amygdala projections may also disinhibit PAG neurones, especially in fearful situations. Most RMN neurones are serotonergic neurones, eventually leading to excitation/inhibition depending upon the array of receptor sub-types in target neurones. They reach the ipsilateral dorsal horn via the dorso-lateral funiculus, and tend to make synapses onto enkephalinergic inhibitory neurones. When activated, these inhibitory interneurones exert a pre- and post-synaptic inhibition onto the dorsal horn projection neurones. Other possible

effects, consequent to the activation of the inhibitory descending system, are consistent with a direct inhibition due to the release of inhibitory neurotransmitters, resulting in: (i) inhibition of neurotransmitter release from noci-responsive PAF terminals; (ii) inhibition of local excitatory interneurons; and (iii) direct inhibition of spinal projection neurones (Merighi *et al.*, 2008).

2.3.3.2 Descending facilitatory pathways

Facilitatory neurones are reported to originate from medullary nucleus reticularis, in the parabrachial nucleus, in the cortex and in other brain areas descending in the ventral/ventrolateral spinal cord and relayed most importantly through RMN and the medial nucleus raphe magnus (Gebhart, 2004). In general, descending inhibitory influences are predominant and often tonically active, whereas facilitatory influences are induced directly by noxious stimuli, probably in order to maintain the hyperalgesic state and, consequently, protect the damaged tissues (Gebhart, 2004).

Rostral ventral medulla (RVM) and descending modulation of pain: There are three types of cells found in the RVM, and they have been described as on-, off- and neutral cells (Porreca *et al.*, 2002; Marchand, 2008). The off-cells are tonically active and pause in firing immediately before withdrawal from a noxious stimulus, while the on-cells accelerate firing immediately before the nociceptive reflex occurs. Neutral cells are initially characterized by the absence of a response to noxious stimulation (Porreca *et al.*, 2002). The activation of OFF-cells correlates with inhibition of nociceptive input and nocifensive responses, while the ON-cells increase firing just before a nociceptive reflex (Schaible, 2006).

2.4 Pain Transduction

Pain transduction is defined as the responses of peripheral nociceptors to noxious or non-noxious chemical, thermal, or mechanical stimuli. It involves conversion of noxious stimuli into calcium ion (Ca^{2+}), mediated electrical depolarisation within the nociceptor terminals. Noxious mediators are either released peripherally from the damaged cells or as a result of humoral and neural responses to the injury, which are usually associated with the release of intracellular hydrogen (H^+) and potassium (K^+) from lysed cell membranes. Prostaglandins and intracellular H^+ and K^+ ions play crucial roles as primary activators of peripheral nociceptors (Vadivelu, 2006). Nociceptor activation initiates a depolarizing Ca^{2+} current or generator potential. The generator potentials depolarize the distal axonal segment and initiate an inward Na^+ current and self-propagating action potential.

2.5 Contribution of Ion Channels in Pain Signaling

Pain occurs as a result of tissue injury in peripheral receptors of sensory neurones (Todorovic and Todorovic, 2006). Electrical impulses are usually generated from the site of the injury, which are transmitted to higher centres in the central nervous system (CNS). The CNS generates protective responses of self-preservation (Todorovic and Todorovic, 2006). Sensory nerve endings express chemo-, mechano-, and thermo-sensitive ion channels, which include acid-sensitive ion channels (ASIC), degenerin/epithelial sodium channels (DEG/ENAC), adenosine triphosphate (ATP)-gated ion channels (P2X), voltage-gated calcium channels, voltage-gated sodium channels, N-Methyl-D-aspartate (NMDA) receptors, Ca^{2+} permeable AMPA, kainate receptors and transient receptor potential (TRP) channels (Premkumar and Sikand, 2008; Xu and Duan, 2009).

Damage as a result of injury to the sensory pathways (nociceptors) may result in a reduced ability to sense pain, or may lead to spontaneous pain and heightened pain sensitivity. Neuronal excitability of nociceptors' activity may be modulated by a number of different voltage-gated ion channels (Todorovic and Todorovic, 2006).

2.5.1 Voltage-gated calcium channels

Calcium plays a crucial role in pain transmission and signaling, a large body of evidence implicates various types of calcium permeable ions in neuronal excitability and synaptic transmission in the nociceptive sensory pathway, and are also important for the induction and expression of pain hypersensitivity (Xu and Duan, 2009). Voltage-gated calcium channels are found in all excitable cells. It is required for neurotransmitter release, and it regulates many aspects of neural activities from neuronal excitability to intracellular changes including gene expression as cell survival and death (Ogasawara *et al.*, 2001; Cao, 2006; Rahman and Dickenson, 2013; Kerkhove *et al.*, 2014).

Voltage-gated calcium channels are broadly categorised into two: high-voltage-activated (HVA), including L-, N-, P/Q- and R-type Ca^{2+} channels that require strong depolarisation for activation, and low-voltage activated (LVA) T-type Ca^{2+} channels that can be triggered by much lower depolarisation (Cao, 2006). Ten subtypes have been identified and further classified as L ($\text{Cav}1.1\text{--}1.3$), N ($\text{Cav}2.2$), P/Q($\text{Cav}2.1$), and R-type ($\text{Cav}2.3$ and T-type ($\text{Cav}3.1\text{--}3.3$) (Rahman and Dickenson, 2013).

The N-type (Cav2.2) is of particular interest since it is concentrated in laminae I and II, of the superficial dorsal horn, where nociceptive primary afferents synapse. The T-type calcium channels (Cav3.1–3.3) are activated at low thresholds, close to resting membrane potential, and thus, play an important role in nociceptive signaling at all levels from the periphery to the brain. An increase in T-type currents has been associated with decreased nociceptive threshold (Rahman and Dickenson, 2013). The P/Q-type channel is suggested to participate critically in neural excitability, integration, and Ca²⁺-dependent neurotransmitter release, based on their expression on somato-dendritic membranes and presynaptic terminals of most central and peripheral neurones (Ogasawara *et al.*, 2001).

2.5.2 Voltage-gated sodium channels

Sodium and voltage gated sodium channels are essential for the initiation, propagation and conduction of action potentials in excitable tissues (Rahman and Dickenson, 2013; Lee *et al.*, 2014). Transduction of tissue-damaging and chemical stimuli and transmission will depend on sodium channels, indicating them as important targets in pain management (Rahman and Dickenson, 2013).

Sodium gated ion channels are classified according to their sensitivity to tetrodotoxin (TTX) into nine homologous subtypes (Nav1.1 – Nav 1.9). Each of which plays specific roles in several physiological processes and development of diseases (Baker and Wood, 2001; Lee *et al.*, 2014). The voltage-gated sodium channels are further categorized in three main groups based on sequence and function. The first group comprises four neuronal sodium channels (Nav1.1, Nav1.2, Nav1.3 and Nav1.7) that are sensitive to tetrodotoxin (TTX-S).

Nav1.1 and Nav1.2 are located in the CNS, Nav1.7 is found peripherally and Nav1.3 is more widely distributed (Rogers *et al.*, 2006). The second group is resistant to TTX (TTX-R) and consists of Nav1.5 found in cardiac muscle and Nav1.8 in nociceptive neurones. The third is an intermediate group, which includes Nav1.4 and Nav1.6 expressed in skeletal muscle, central and peripheral axons, respectively (Rogers *et al.*, 2006). Nav1.1 and 1.6-1.9 are expressed in high levels in dorsal root ganglia (DRG) (Rahman and Dickenson, 2013). Most importantly, Nav1.7, Nav1.8 and Nav1.9 contribute significantly to inflammatory pain processing, where they serve as a threshold channel and promote hyperexcitability to sub-threshold stimuli (Rahman and Dickenson, 2013). Mutation in SCN9A which codes for Nav1.7 results in inability to perceive pain in humans (Lee *et al.*, 2014). Genetic mutations in the SCN9A gene that encodes the Nav1.7 sodium channel α subunit results in congenital insensitivity to pain (Rahman and Dickenson, 2013).

2.5.3 Acid sensing ion channels (ASIC)

Acid sensing ion channels are Na⁺ channels, activated by external protons and responding to a reduction in extracellular pH (Deval *et al.*, 2010). They share about 20-25% similarity to a larger Na⁺ channel family, known as epithelial/degenerin Na⁺ channels (ENaCs). Six types (iso-forms) of ASIC family have been identified and encoded by four different genes: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 (Bronstein-Sitton, 2004). Among them, only ASIC3 and ASIC1b to a lesser degree are linked to pain perception (Bronstein-Sitton, 2004; Gohar, 2005).

2.5.4 Transient receptor potential channels

The superfamily of mammalian transient receptor potential (TRP) channels consists of approximately 30 proteins, divided into six subfamilies: ankyrin (TRPA), canonical, melastatin (TRPM), mucolipin, polycystin, and vanilloid (TRPV). Among TRP channels, nine are highly sensitive to temperature and are referred to as the thermo-TRP channels. They include the heat-activated TRPV1 to TRPV4, TRPM2, TRPM4, and TRPM5 as well as the cold-activated TRPA1 and TRPM8 (Romanovsky *et al.*, 2009). Amongst them, the TRPV1 (vanilloid 1) channel is best known for its role in nociception and sensory transmission (Ho *et al.*, 2012; Martins *et al.*, 2014).

2.6.4.1 TRPV1 receptors

TRPV1, originally named vanilloid receptor 1 (VR1) and are commonly referred to as the capsaicin receptors. TRPV1 was identified by expression cloning using the 'hot' pepper-derived vanilloid compound, capsaicin as a ligand (Clapham, 2002). It is a polymodal receptor that is activated by vanilloid compounds (capsaicin, resiniferatoxin), moderate heat (≥ 43 °C) and low pH (< 5.9) (Levine and Haber, 2007; Fernandes *et al.*, 2012). TRPV1 was cloned by Catarina and colleagues in 1997 (Catarina *et al.*, 1997). TRPV1 is a Ca^{2+} permeant non-selective cation channel expressed predominantly by unmyelinated C-fibres and thinly myelinated A δ fibres and plays a major role in inflammatory thermal sensation. TRPV1 is activated by heat ($\sim > 43^\circ\text{C}$), protons, N-arachidonyl dopamine (NADA), anandamide and lipoxygenase metabolites of arachidonic acid (Pabbidi *et al.*, 2008). Inflammatory mediators (bradykinin, prostaglandin E2, extracellular ATP, glutamate and nerve growth factor) indirectly sensitize TRPV1. Inflammatory mediators sensitize TRPV1 function by various

mechanisms; they may increase TRPV1 expression levels in the membrane, induce TRPV1 phosphorylation by protein kinases or release the inhibition of TRPV1 by phosphatidylinositol 4,5-bisphosphate, which render the channel more responsive to agonist stimulation. In addition, these inflammatory mediators activate TRPV1 receptors that are coupled to G proteins or tyrosine kinase pathways; thus, activating phospholipase C and/or phospholipase A₂ which, in turn, induce the release of arachidonic acid metabolites (Ramsey *et al.*, 2006; Patapoutian *et al.*, 2009).

The TRPV1 initiates nociceptive signalling by creating a generator or receptor potential at the peripheral nerve ending, through increased membrane permeability to non-selective cationic current (monovalent and divalent cations) especially Ca²⁺ and, consequently, membrane depolarisation (Premkumar and Sikand, 2008; Romanowsky *et al.*, 2009; White *et al.*, 2010) TRPV1 has since been described in many other neuronal and non-neuronal cells, its highest expression level is in afferent sensory neurones (Riera *et al.*, 2014). Repeated administration of TRPV1 agonist, or prolonged exposure of TRPV1 receptor, to capsaicin or resinaferatoxin results in desensitisation (Fernandes *et al.*, 2012); thus, conferring analgesic properties. TRPV1 pain receptors have been reported to prolong lifespan, in a study that observed that mice lacking TRPV1 pain receptors lived longer than mice, which had TRPV1 pain receptors by 11.9% and 15.9% in male and female mice, respectively (Riera *et al.*, 2014). This appears to be mediated by inactivation of cAMP response element-binding protein/ CREB-regulated transcription co-activator 1 (CRTC1/CREB) pathway in sensory neurones. Absence of TRPV1 pain receptors also protects against diet-induced obesity suggesting its role in metabolism (Riera *et al.*, 2014).

2.6 Pain and Circadian Rhythms

Pain is a complex phenomenon that is influenced by many factors that includes; anxiety, fatigue, prior experiences, season and circadian rhythm. Day patterns and seasonal variability have been demonstrated to affect pain intensity, pain perception and use of medication (Bruguerolle and Labrecque, 2007). Pain is rarely constant over the 24-hour period; rather, it is circadian-time dependent (Bruguerolle and Labrecque, 2007). Chronobiology can be defined as the study of rhythmic patterns in biological phenomena (Manfredini *et al.*, 1998). Chronopharmacology and chronotherapeutics which are related to chronobiology are recent scientific fields that study when drugs are most effective and best tolerated (Kusunose *et al.*, 2010).

Life is intimately linked to temperature, and a living organism is constantly responding to changes in ambient and body temperatures with a variety of physiological and behavioral responses (Romanowsky *et al.*, 2009). It is known that ambient temperature influences pain perception, but it is unknown the extent to which it influences pain. Pain is usually accompanied by structural and functional changes (plasticity). In fact, recent animal and clinical studies demonstrate that epigenetic influence in pain occurs, possibly by modulating the transition of acute pain to chronic pain (Bai *et al.*, 2014).

2.6.1 Circadian variation in pain neurochemistry

Several studies have demonstrated circadian patterns in the brain levels of pain neurochemicals like opioid peptides, 5-hydroxytryptamine, bradykinin, glutamate, nitric oxide (NO), substance P, cytokines (interleukin-1(IL1), interleukin-6 (IL6) and prostanoids.

For instance, brain concentrations of substance P are circadian time-dependent in rats, with highest values found during their nocturnal activity span (Kelderlhye *et al.*, 1981). Circadian variations in the concentration of β -endorphin have also been reported to peak late in the rest period of the rats (Wesche *et al.*, 1979). Although Finn *et al.* (2006) reported a contrary finding that rat β -endorphin concentrations in the brain are not affected by diurnal variation., suggesting that in mice, analgesia is higher at night than day time (Finn *et al.*, 2006).

2.7 Pain and Oxidative Stress

Reactive oxygen and nitrogen species (ROS, RNS, respectively) are highly reactive molecules produced during normal metabolism and are derived from oxygen, and nitrogen. They include free radicals like superoxide ($O_2^{\cdot-}$) and hydroxyl radical (HO^{\cdot}) and other reactive species; hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Nishio *et al.*, 2013). Overproduction of ROS induces an imbalance between prooxidants and antioxidants in cells and tissues, resulting in oxidative stress which are associated with numerous disease states (Filaire and Toumi, 2012; Basudhar *et al.*, 2015). ROS concentrations are strictly controlled by antioxidant enzymes. The most essential among them is mitochondrial superoxide dismutase (MnSOD) which converts superoxide to hydrogen peroxide (H_2O_2). Hydrogen peroxide is further converted into molecular oxygen and water by either catalase or glutathione peroxidase (Schwartz *et al.*, 2009).

The ROS and RNS contribute significantly to the development of pain of several aetiologies especially chronic, inflammatory and neuropathic pain (Schwartz *et al.*, 2009; Salvemini *et al.*, 2011; Keilhoff *et al.*, 2013, Nishio *et al.*, 2013). Existing evidence has implicated

superoxide (SO_2^-), peroxynitrite (ONOO^-), NO and other free radicals in the transition of acute to chronic pain (Salvemini *et al.*, 2011). They have also been reported to be actively involved in peripheral and central sensitisation of pain; thus, they are potential targets for therapeutic interventions of pain management (Wang *et al.*, 2008). The involvement of SO_2^- and ONOO^- as crucial pronociceptive mediators have been demonstrated by their direct injection (Siniscalco *et al.*, 2007). ROS and RNS may induce visceral pain, mechanical allodynia, thermal hyperalgesia and regulation of pro-apoptotic gene expression (Siniscalco *et al.*, 2007; Molinga-Ortega *et al.*, 2014). The effects of ROS are seemingly mediated by direct action of superoxide and hydrogen peroxide, and in an indirect manner by the RNS (Kallenborn-Gerhardt *et al.*, 2013). ROS and RNS have also been associated with increased supraspinal, descending facilitation of pain. Although the mechanism is unclear, increased activity of ONOO^- and decreased MnSOD activity have been reported; especially in the RVM, which is a critical centre for pain modulation. In the amygdala, nociceptive facilitation is mediated ROS (Salvemini *et al.*, 2011; Nishio *et al.*, 2013).

Glutamate is the major excitatory neurotransmitter that plays a key role in nociception, synaptic plasticity and development of acute and chronic inflammatory pain. Increased activation of glutamate receptors (NMDAR) increases neuronal excitability consequently leading to increase pain signaling. ONOO^- interacts with the NMDAR leading to constant potentiation of synaptic currents and calcium influx, and ultimately excitotoxicity (Siniscalco *et al.*, 2007). Neuronal apoptosis is an important mediator in the development of hyperalgesia and sensitization, especially in neuropathic pain. The RNS species have been suspected to contribute to pain by stimulating apoptosis but the specific mechanism is still

poorly understood (Salvemini *et al.*, 2011). TRPV1 plays a central role in nociception because it integrates multiple pain stimuli (polymodal) and it is essential for nociceptive signaling. TRPV1 is also implicated in central sensitisation and long-term potentiation through its association with glutamatergic signaling. Studies suggest that sensitisation of TRPV1 results in the production of ROS and RNS, serves as channel for the release of glutamate from presynaptic terminals through its activation (Salvemini *et al.*, 2011; Nishio *et al.*, 2013).

2.8 Pain and Antioxidants

An imbalance between pro-oxidant and antioxidant activities have been observed in inflammatory pain. Therefore, antioxidants are usually administered as adjuncts, allowing the possibility of lowering doses of the drugs and increasing efficacy. In line with this, the analgesic properties of vitamin E have been demonstrated to act through desensitisation and/or inactivation of NMDA receptors (Kim *et al.*, 2006). The use of antioxidants as adjuncts in pain management has been attempted with favourable and effective results (Pezilli and Fantini, 2007). Deficiency in selenium has been associated with increased pain, while vitamins A, C and E supplementation was associated with reduced pain episodes (Canter *et al.*, 2007; Holt, 2014). The advantage of this therapy is low cost, especially in less economically developed countries. Extracts of plants with antioxidant properties and flavonoids (rutin and quercetin) have also been identified to improve pain management (Azevedo *et al.*, 2013; Dzoyem and Eloff, 2015).

2.9 Resveratrol

Resveratrol, 3, 4', 5-trihydroxy-trans-stilbene, is a natural non-flavonoid polyphenol compound containing a stilbene structure similar to that of estrogen, diethylstilbestrol (Robb and Stuart, 2010; Bhullar and Hubbard, 2015). It is also referred to as a phytoalexin because it is secreted in plants in resistance to diseases (Siemann and Creasy, 1992; Magalhaes *et al.*, 2014). It is a fat-soluble compound, existing in *cis*, *trans*-, and piceid isomeric forms. Resveratrol is produced by various plants as a defence against stress, injury, excessive sunlight, ultraviolet radiation, infection and invading fungi. It is also considered a nutraceutical present in grapes, peanuts, pine trees, cassia, grape fruits, red wine and many food products (Carrizo *et al.*, 2013).

Resveratrol was first isolated in 1940 from the roots of white hellebore (*Veratum grandiflorum* O. Loes) and later, in 1963, from the roots of *Polygonum cuspidatum*, a plant used in traditional Chinese and Japanese medicine. Despite its ancient discovery, the first real interest in resveratrol came in 1992, when it was postulated to explain some of the cardio-protective effects of red wine (Siemann and Creasy, 1992). Resveratrol possesses multiple pharmacological potentials that include anti-inflammatory, anti-cancer, immunomodulatory, neuroprotective, free-radical scavenging effects. It also possesses hypoglycaemic and potent antioxidant activities (Torres-Lopez *et al.*, 2002; Lu *et al.*, 2006; Candelario-Jalil *et al.*, 2007; Harb *et al.*, 2009; Cenesiz *et al.*, 2012; Anton *et al.*, 2014; Singh and Pai, 2014; Tamaki *et al.*, 2014). However, the most celebrated effect of resveratrol is its effect on extending life span (longevity) in various organisms from yeast to mammals (Howitz *et al.*, 2003; Bauer *et al.*, 2004; Wood *et al.*, 2004; Viswanathan *et al.*, 2005;

Valenzano *et al.*, 2006). Trans-Resveratrol has an isomer cis-resveratrol with lower bioactivities. Isomerization from trans- to cis-form is thought to be facilitated by UV/sunlight radiation. Resveratrol is an extremely photosensitive compound. It exhibits 80-90% of the trans-resveratrol in solution, which gets converted to cis-resveratrol, if exposed to light for 1 h (Singh and Pai, 2014; Zhao *et al.*, 2015).

In recent times, resveratrol has been criticized for its therapeutic uses because of its low bioavailability, although resveratrol has been consistently reported by dozens of studies to have health benefits. Different strategies have been employed to improve its bioavailability; including encapsulation, which prevents degradation, increasing its solubility in water or using niosomes-containing resveratrol (Pando *et al.*, 2015).

2.9.1 Antioxidant properties of Resveratrol

Antioxidant properties of resveratrol have been well documented in the literature. It protects against oxidative effects of free radicals (Magalhaes *et al.*, 2014; Ozdemir *et al.*, 2014). Resveratrol has been reported to inhibit inducible nitric oxide synthase and NF κ B, thus reducing NO generation (Ozdemir *et al.*, 2014). It is a potent scavenger of free radical (Singh and Pai, 2014). One of the mechanisms suggested to underlie the antioxidant properties of resveratrol is the activation of the nuclear factor E2-related factor 2 (Nrf2)/antioxidant defence pathway. Nrf2 is an important sensor of toxic xenobiotic substances and oxidants and its activation is a crucial cellular defense mechanism. Upon activation, Nrf2 dissociates from its cytoplasmic repressor Kelch-like ECH-associated protein and translocates to the nucleus, where it interacts with the antioxidant defense to mediate the transcription target

genes such as haemeoxygenase1(HO-1) and NAD(P)H, quinone oxido-reductase1(NQO-1). The transcription of these genes enhances cellular resistance to oxidative stress and confers protection against inflammation (Tamaki *et al.*, 2014).

2.9.2 Resveratrol and pain

Studies have demonstrated that resveratrol possesses antinociceptive properties (Granados-soto *et al.*, 2002). Resveratrol inhibits hyperalgesia induced by formalin injection in rats (Gentili *et al.*, 2001; Torres-Lopez *et al.*, 2002). Pain modulation involves K⁺ channels; activation of K⁺ channels leads to antinociception, while blocking of K⁺ channels reduces antinociception, especially when morphine is administered (Granados-Soto, 2002). One of the mechanisms by which resveratrol exerts its antinociceptive action is the modulation of K⁺ ion channels in peripheral afferent neurones. Another underlying mechanism of the analgesic activity of resveratrol is inhibition of Na⁺ ion channels in dorsal root ganglia (Kim *et al.*, 2005). Tolerance to morphine has been demonstrated to be reversed by resveratrol through activation of SIRT1 (He *et al.*, 2014). Although there are reports that resveratrol has no thermal antinociceptive activity, chemical antinociception has been documented (He *et al.*, 2014; Pharm-Macou *et al.*, 2008) via inhibition of cyclooxygenase cascade.

CHAPTER THREE

3.1 Materials and Methods

3.1.1 Chemicals and drugs

Resveratrol (Candlewood Stars Incorporated, Danbury, USA, Batch Number: MR 150528), Carboxymethyl cellulose (Product No; 27929, BDH Laboratory Chemicals Limited Poole, England) and formalin was obtained from the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria.

3.1.2 Equipment

Randall-Selitto analgesiometer (No: 92471, Ugo-Basile, Milan-Italy), electric hot-plate (DB-1A, Life Assistance Scientific United Kingdom, cages, mortar and pestle, weighing balance, syringes, oral cannulas, ethylenediaminetetraacetic acid (EDTA) bottles, sample bottles, dissecting kits, stop watch, wet- and dry-bulb thermometers.

3.2 Resveratrol Preparation and Administration

Trans-resveratrol, was suspended in 10 g/L carboxymethyl cellulose (CMC), because it is poorly soluble in water and then administered orally (Juan *et al.*, 2005).

3.3 Experimental Animals and Management

A total of 100 young adult Wistar rats (50 males, 50 nulliparous females) with an average weight of 125.33 ± 2.66 g and 7-9 weeks of age were used for this study. The rats were housed in cages (5 rats in each). Each rat was tested once only and efforts were made to minimise animal suffering.

3.3.1 Animal groupings and Experimental protocol

The animals were divided into male and female groups (Fig. 3.1). Each group was further assigned into four subgroups of five animals each, as follows:

Group 1 (Control): Rats in this group were given distilled water and exposed to noxious stimulation.

Group 2 (CMC): Rats in this group were given carboxymethyl cellulose (CMC) orally (which was used a vehicle for resveratrol because it is poorly soluble in water) and thereafter exposed to noxious stimulation.

Group 3 (RSV): Rats were first administered resveratrol (200 mg/kg) and exposed to noxious stimulation (Xu *et al.*, 2009).

Group 4 (Naïve): Rats were not exposed to any noxious stimulus and were not administered with any drugs (they were used for comparison in the biochemical assessment)

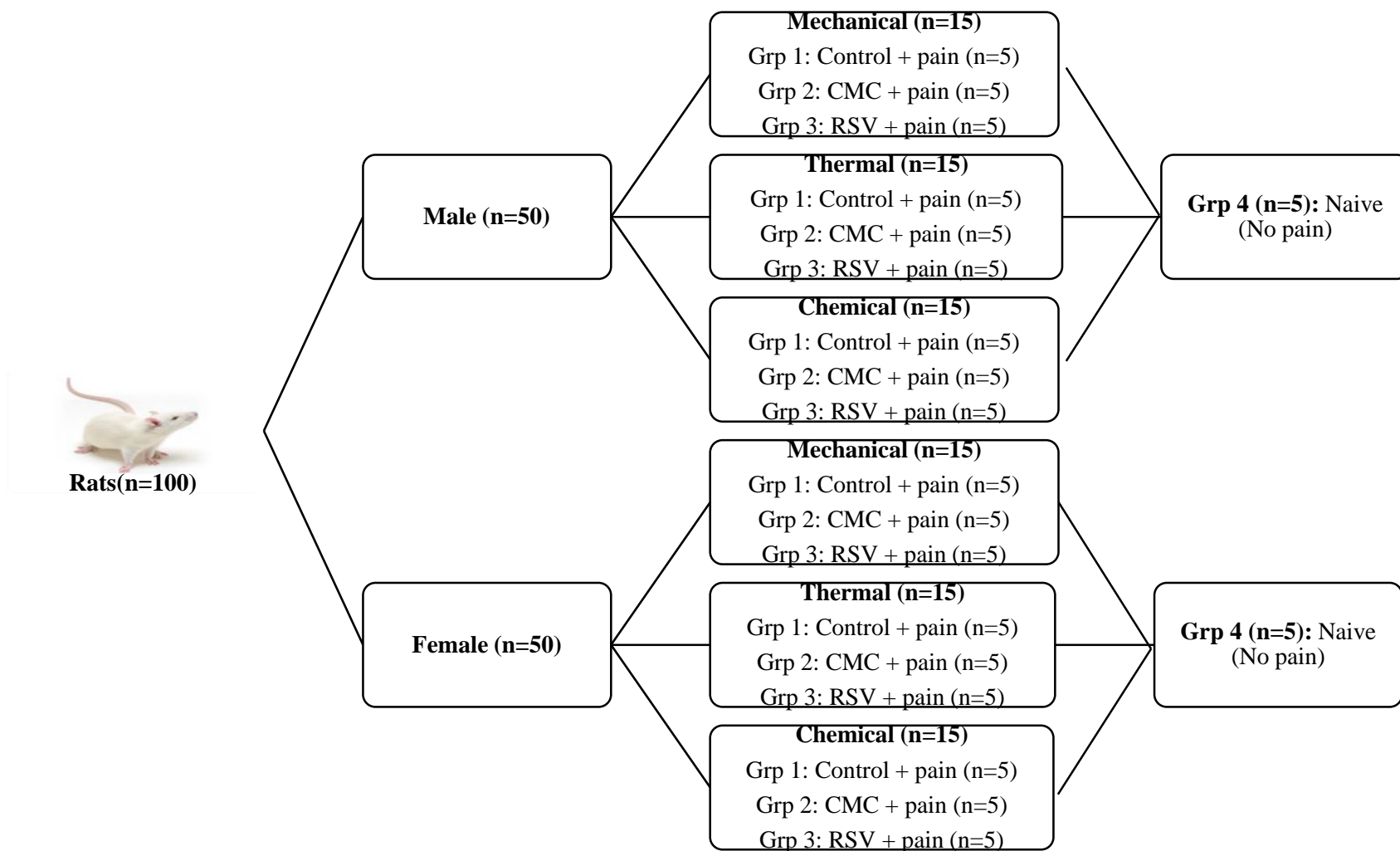


Figure 3.1: Experimental groupings, Grp = Group, CMC = carboxymethyl cellulose, RSV = resveratrol.

3.4 Methodology

3.4.1 Measurement of thermal environmental parameters

Environmental temperature and relative humidity were recorded during the Harmattan period (November-February) using the dry- and wet-bulb thermometer, and according to the manufacturer's instruction. The readings were taken at 6:00h, 13:00h and 18:00h. Heat index (HI) was calculated using the product of dry bulb thermometer reading and relative humidity according to Jackson (2009).

$$\begin{aligned} \text{HI} = & -42.379 + 2.04901523T_F + 10.14333127\text{RH} \\ & - 0.22475541T_F \text{RH} - 6.83783\text{E} - 3T_F^2 \\ & - 5.481717\text{E} - 2\text{RH}^2 + 1.22874\text{E} - 3T_F^2\text{RH} \\ & + 8.5282\text{E} - 4T_F \text{RH}^2 - 1.99\text{E} - 6T_F^2 \cdot \text{RH}^2(^{\circ}\text{F}). \end{aligned}$$

3.4.2 Pain sensitivity tests

3.4.2.1 Paw pressure test

Paw pressure test used as index of mechanical nociception according to the method of Randall and Selitto (1957), with modification by Woode *et al.* (2013). An analgesiometer (Ugo Basile Biological Research Apparatus, SN: 15776 v 220, Milan, Italy) was used to apply linearly, increasing mechanical force (pressure) by means of the tip of a blunt perspex cone to the dorsal region of the right hind paw, until the animal exhibited nociceptive withdrawal. The nociceptive withdrawal threshold was measured by recording the distance covered on the analgesiometer scale at which each mouse exhibited nociceptive withdrawal. The intensity of force (in g) causing an escape reaction was defined as the withdrawal threshold. The nociceptive withdrawal threshold was measured at 9:00 h, 13:00 h and 18:00 h (GMT +1) post-drug administration. The maximum force applied was limited to 250 g

(2500N) to prevent tissue damage. The results were recorded as latency (reaction time) and a maximum cut-off (20 s) was set up to avoid prolonged nociception and tissue damage.

3.4.2.2 Hot-plate test

The hot-plate test was used to measure pain sensitivity to acute thermal noxious stimulus, described and modified by Wolf *et al.* (2003). Briefly, rats were injected with resveratrol (test) or CMC (controls) and then placed on the hot-plate one at a time after 60 minutes of administration. Latency to respond to the heat stimulus was measured by the amount of time it took for the animal to lick one of its paws or jump response. In order to avoid tissue damage, the maximum time the animal was allowed to spend on the hot plate was 60 seconds.

3.4.2.3 Formalin Test

Formalin test as described by Dowling *et al.* (2009) and modified by Yerima *et al.* (2009) was used to evaluate pain response to chemical pain. Briefly, rats were administered distilled water, CMC, or resveratrol (200 mg/kg) orally. After 1 hour, 50µl of freshly prepared 2.5% formalin solution was injected subcutaneously into the dorsal surface of the right hind paw of the animal using a syringe with a 26-gauge needle. The animal was gently restrained with the hand, while the paw was injected. After the injection, the rat was immediately placed back into the observation chamber. Subcutaneous injection of formalin produces a biphasic neuronal and behavioural response, consisting of an early, short-lasting phase and a late, long-lasting tonic phase (Eisenberg, 1993). The initial acute or early phase occurred during the first 5 minutes, which is then followed by a prolonged tonic response or late phase during the last 45 minutes with a 10 minutes' lag period in-between both phases.

The severity of pain response was recorded for each rat based on the following scale: (0) rat walked or stood firmly on the injected paw; (1) the injected paw was favoured or partially elevated; (2) the injected paw was clearly lifted off the floor; (3) the rat licked, chewed or shook the injected paw.

3.5 Biochemical Assessment

After completion of each pain assessment, all rats were humanely sacrificed. Blood samples were collected in EDTA bottles and centrifuge tubes and processed for the following biochemical analyses.

3.5.1 Assessment of electrolyte concentration

3.5.1.1 Sodium ion assessment

Serum sodium was measured by colorimetry method of Maruna (1951) and Trinders (1958). Briefly, 1000 μL of precipitation reagent (Sodium R1), 10 μL of sodium and 10 μL of serum were mixed vigorously and allowed to incubate at room temperature for 5 minutes. Then centrifuged at 3000 RPM to obtain clear supernatant. Supernatant was transferred for standard and test, assay and the absorbance measured at 530 nm.

3.5.1.2 Potassium ion assessment

Serum potassium was estimated by colorimeter method of Henry (1974) and Tietz (1970). Briefly, 1000 μL of potassium reagent, 25 μL of standard and 25 μL of serum were mixed well and allowed to incubate at room temperature for 5 minutes. Then centrifuged at 3000 RPM to obtain clear supernatant. The supernatant was transferred for standard and test assay and the absorbance was measured at 530 nm against distilled water.

3.5.1.3 Chloride ion assessment

Serum chloride was estimated by colorimetry method of Schonfeld and Lowellen (1964). Briefly, 1000 μL of chloride reagent, 10 μL of standard and 10 μL of serum were mixed well and allowed to incubate at room temperature for 1 minutes. The absorbance of the standard and test samples was measured against distilled water.

3.5.2 Erythrocyte osmotic fragility test

The EOFT was done as described by Faulkner and King (1970). Briefly, sodium chloride (NaCl) solution was prepared in volume of 500 mL for each of the samples and in concentrations, ranging from 0.0 to 0.9% at pH 7.4. A set of 10 test tubes, each containing 10 mL of NaCl solution of varying concentrations, ranging from 0.0 to 0.9% was arranged serially in a test tube rack. One set was used to analyse each sample. The test tubes were labelled with corresponding NaCl concentration. One millilitre pipette was used to transfer 0.02 mL of blood sample into each of the 6 test tubes. Mixing was done by gently inverting the test tubes for about 5 times. The test tubes were allowed to stand at room temperature (27°C) for 30 minutes. Thereafter, the contents of the test tubes were mixed and centrifuged at $1,500 \times g$ for 15 minutes. The supernatant of each test tube was transferred into a cuvette. The concentration of haemoglobin in the supernatant solution was measured using a spectrophotometer at 540 nm by reading the absorbance. The same procedure was repeated for every blood sample obtained from each rat used for the study.

The percentage haemolysis was calculated using the formula (Faulkner and King, 1970):

$$\text{Percentage haemolysis} = \frac{\text{Optical density of test}}{\text{Optical density of distilled water}} \times 100$$

3.5.3 Assessment of lipid peroxidation

The level of thiobarbituric-acid reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated. Quantitative measurement of MDA was determined using GenAsia Biotech MDA assay kit (Catalogue number: GA-E0164RT, Specificity: Rat Malondialdehyde, Sensitivity: 0.01nmol/ml, Assay range : 0.05nmol/ml-10nmol/ml). The principle is based on the reaction of MDA with thiobarbituric acid (TBA), forming an MDA-TBA adduct that absorbed strongly at 532 nm (Janero, 1990). Quantitative analysis was carried out according to manufacturer's instructions.

3.5.4 Superoxide dismutase activity

Activity of SOD in the rat serum was determined using GenAsia Biotech SOD assay kit (Catalogue number: GA-E0176RT, Specificity: Rat Superoxide dismutase activity, Sensitivity: 0.016ng/ml). The assay kit is based on the principle of superoxide inhibition of autooxidation of hematoxylin as described by Martin *et al.* (1987). Quantitative analysis was carried out according to manufacturer's instructions.

3.6 Data Analyses

Data were expressed as the mean \pm standard error of mean (mean \pm SEM.). Statistical analyses were performed using SPSS (Version 20). Two way mixed analysis of variance (ANOVA) was used to analyse nociceptive response to mechanical and thermal noxious stimulus (time \times treatment \times sex), followed by Tukey's *post-hoc* test. Biochemical assessments were compared using a two way (treatment \times sex) ANOVA. Kruskal Wallis was used to compare nociceptive responses of rats to chemical noxious stimulation. Values of $p \leq 0.05$ were considered statistically significant.

CHAPTER FOUR

4.0 Results

4.1 Thermal Environmental Conditions

Thermal environmental data recorded in the room where the animals were kept during the study period is presented in Table 4.1. The dry-bulb temperature, relative humidity and heat index obtained varied significantly ($p < 0.001$) with the time of measurement, the value at 6:00 h being lower than that obtained at either 13:00 h or 18:00 h. Overall mean dry-bulb temperature, relative humidity and heat index were 21.91 ± 0.44 °C, 18.46 ± 1.27 % and 20.25 ± 0.49 , respectively.

Table 4.1: Thermal environmental data during the study period (Mean \pm SEM)

| Time (h) | Dry Bulb Temperature (°C) | Relative Humidity (%) | Heat Index (HI) |
|---------------------|--|--|--|
| 6:00 | 18.36 \pm 0.62 ^a (15-24) | 26.42 \pm 2.60 ^a (8.7-53.6) | 16.41 \pm 0.67 ^a (13.0-23.0) |
| 13:00 | 23.28 \pm 0.59 ^b (19-29) | 16.17 \pm 1.65 ^b (4.40-36.6) | 21.95 \pm 0.63 ^b (17.0-27.0) |
| 18:00 | 23.17 \pm 0.61 ^b (19-30) | 14.86 \pm 1.78 ^b (2.10-39.4) | 21.67 \pm 0.67 ^b (17.0-28.0) |
| Overall Mean | 21.91 \pm 0.44 | 18.46 \pm 1.27 | 20.25 \pm 0.49 |

Values in parentheses are minimum – maximum. ^{a,b} = mean values with superscript letters are significantly ($p < 0.05$) different.

4.2 Nociceptive Response to Mechanical Noxious Stimulus

The values obtained in rats following responses to mechanical noxious stimulus are shown in Figure 4.1 - 4.3. There was no significant difference in pain responses to mechanical pain stimulus between sexes and treatment groups [Sex: $F(1, 24) = 0.55$; $p = 0.47$; Treatment: $F(2, 24) = 1.45$; $p = 0.255$]. Although a significant [Time: $F(2, 48) = 35.8$; $p < 0.001$] difference was recorded in pain responses at different hours of recording, being highest in the morning (09:00 h) and lowest in the evening (18:00 h). There was no significant interaction between Treatment \times sex [$F(2, 24) = 0.28$; $p = 0.76$], Time \times treatment [$F(4, 48) = 0.714$; $p = 0.59$], Time \times sex [$F(2, 48) = 0.047$; $p = 0.954$], Time \times treatment \times sex [$F(4, 48) = 0.313$; $p = 0.868$]

The highest and lowest mean values of pain responses in control male and female were recorded in the morning and evening ($p < 0.05$), respectively. Values obtained in male and female groups were also lowest at 09:00 h but increased progressively at 13:00 h and finally peaked at 18:00 h ($p < 0.001$). Resveratrol did not significantly modulate mechanical pain threshold ($p > 0.05$) in both sexes and different time periods.

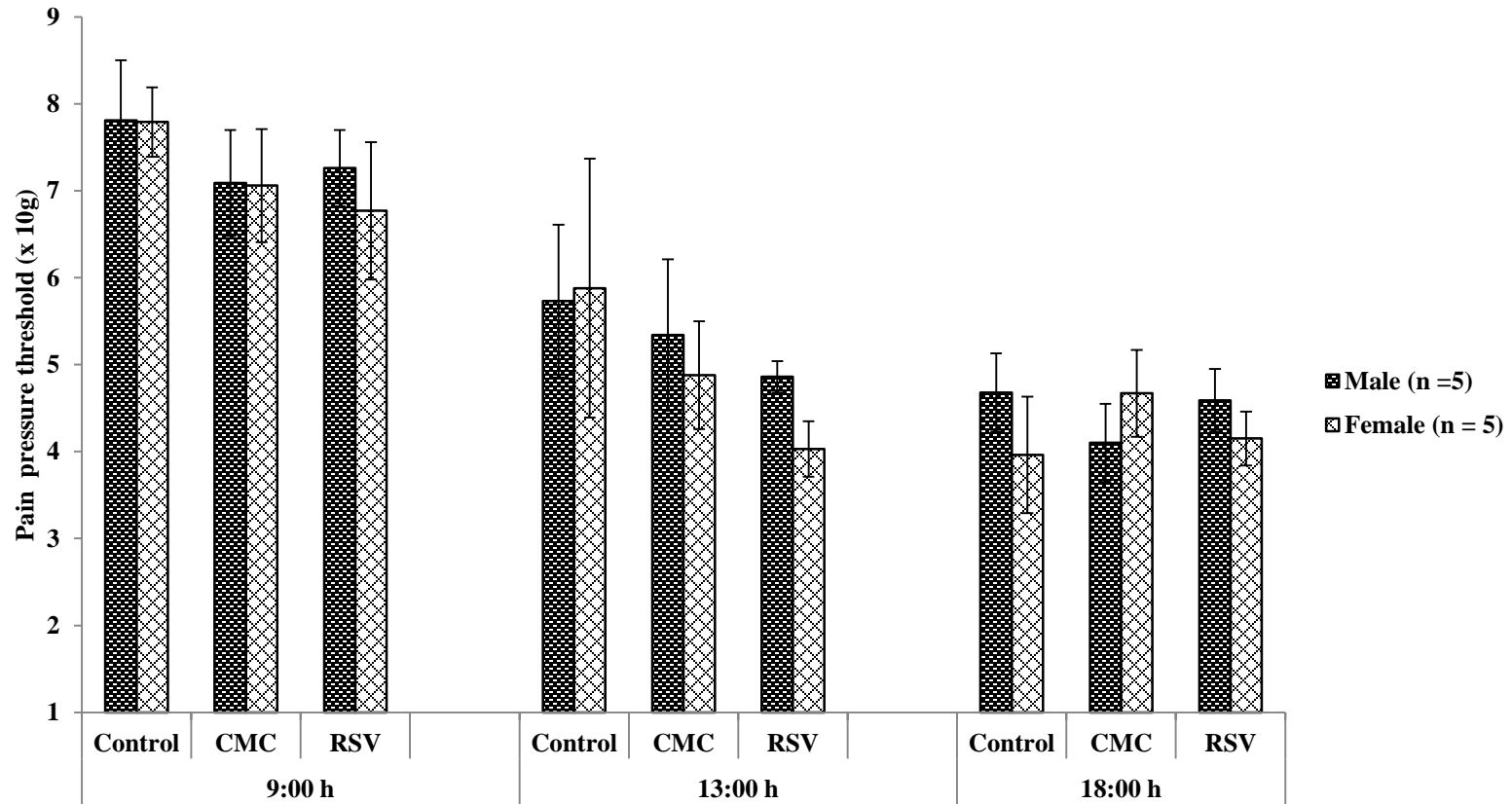


Figure 4.1: Diurnal and sex variations in pain-threshold to mechanical noxious stimulus in Wistar rats during the harmattan season. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

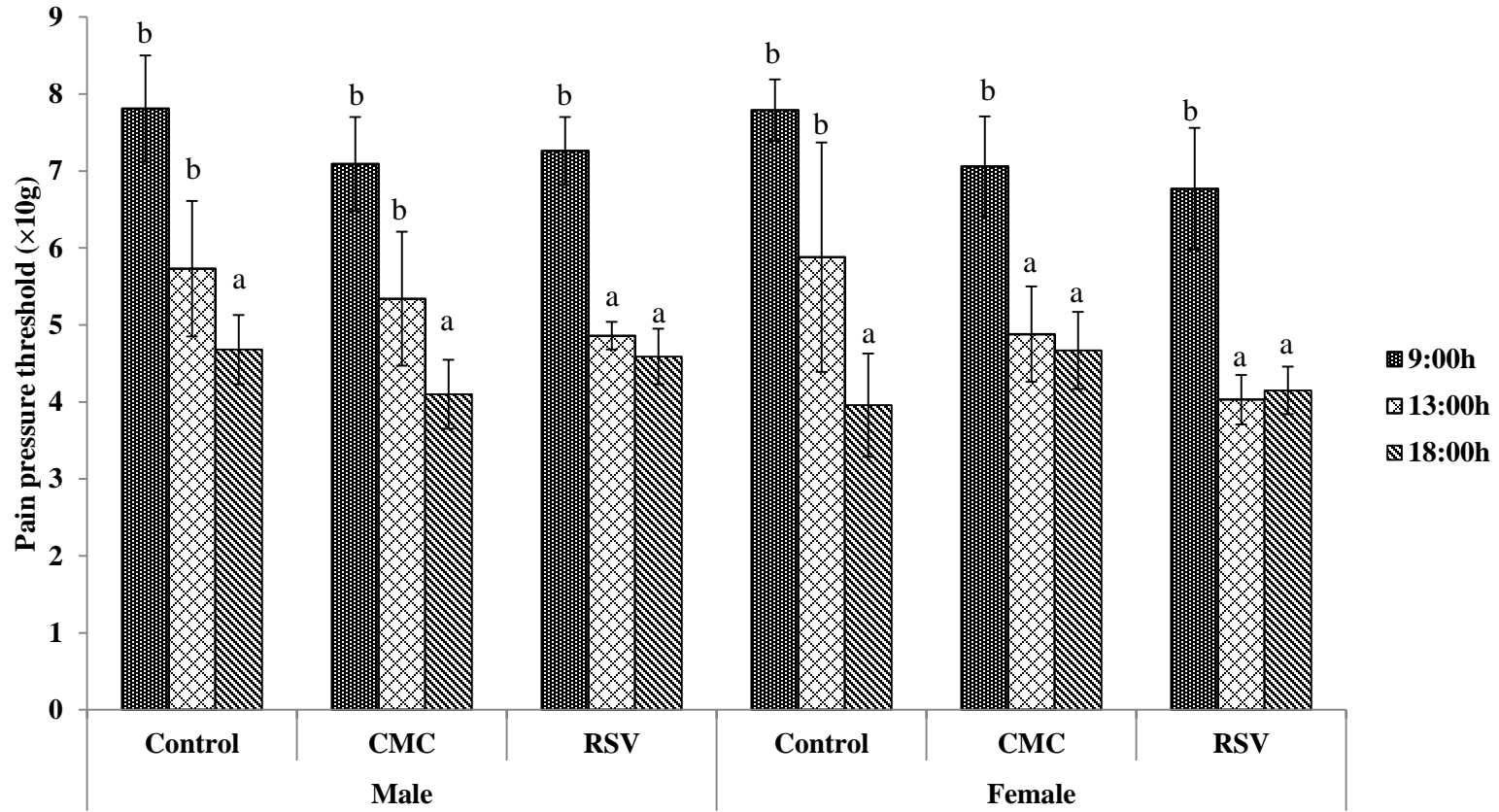


Figure 4.2: Time fluctuations and sex variations in pain threshold to mechanical noxious stimulus in Wistar rats during the harmattan season. CMC = Carboxymethyl cellulose, RSV = Resveratrol. ^{a,b} = means with superscript letters are significantly ($p < 0.05$) different.

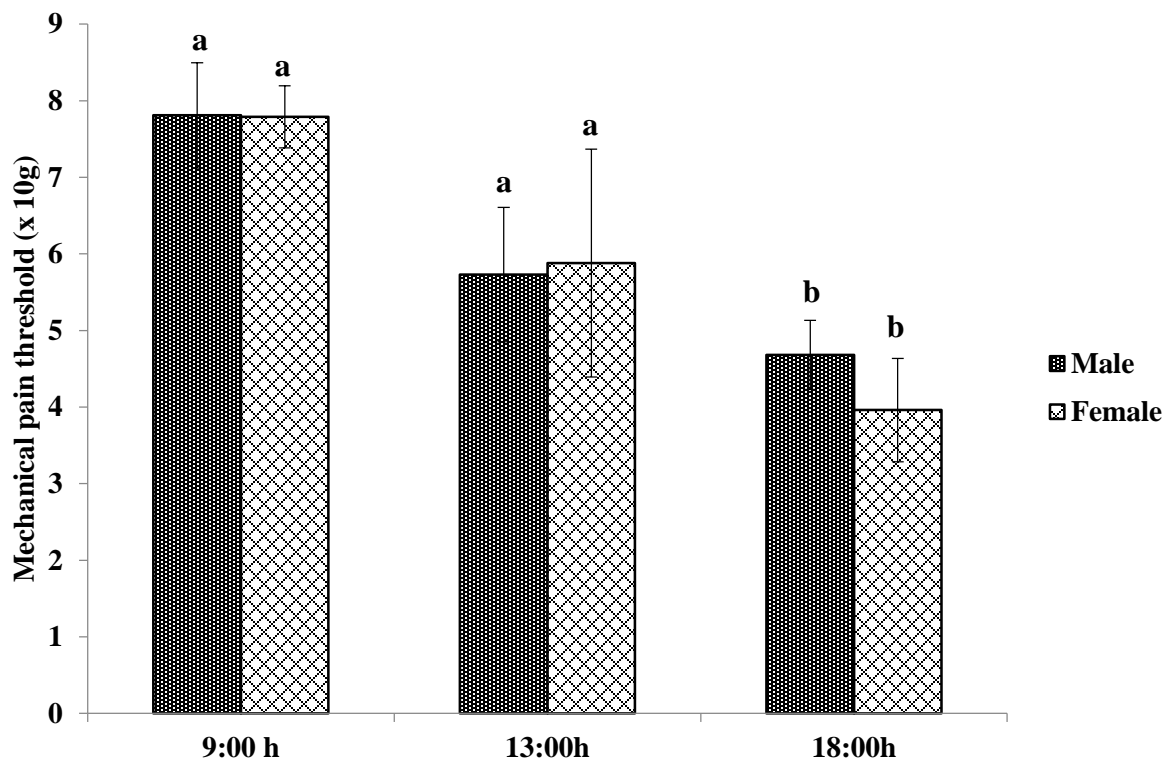


Figure 4.3: Diurnal and sex variations in pain-induced mechanical stimulus in control animals during the harmattan season. Different superscripts ^{a,b} indicate significant difference $p < 0.05$.

* = male vs. female ($p < 0.05$).

4.3 Nociceptive Response to Thermal Noxious Stimulus (Hot-plate test)

No significant difference was recorded in thermal threshold in the treated groups [$F(2, 24)=1.339$; $p = 0.281$], when compared to the controls. Time influenced thermal pain threshold [$F(4, 96)=12.47$; $p = 0.0001$], with jumping latency (seconds) on the hot plate increasing from 0 minutes to 120 minutes (Figure 4.4). Sex did not significantly influence thermal threshold [$F(1, 24)= 0.689$; $p = 0.415$]. There was no interaction of Treatment \times sex [$F(2, 24) = 0.647$; $p = 0.533$], Time \times treatment [$F(8, 96)=0.504$; $p = 0.850$], Time \times sex [$F(4, 96)= 1.286$; $p = 0.281$], Time \times treatment \times sex [$F(8, 96) =0.544$; $p = 0.821$] in this study.

Tables 4.2: Dynamics of time variation in time-course of thresholds of response to thermal pain (hot-plate) stimulus in male and female Wistar rats during the harmattan season.

| Time (Minutes) | | Control | CMC | RSV |
|-----------------------|---------------|----------------|-------------|-------------|
| 0 | Male | 1.11 ± 0.12 | 1.22 ± 0.17 | 1.11 ± 0.16 |
| | Female | 1.31 ± 0.17 | 1.31 ± 0.13 | 1.12 ± 0.12 |
| 60 | Male | 1.45 ± 0.28 | 1.37 ± 0.14 | 1.40 ± 0.12 |
| | Female | 1.45 ± 0.13 | 1.30 ± 0.08 | 1.19 ± 0.07 |
| 90 | Male | 1.64 ± 0.16 | 1.54 ± 0.10 | 1.44 ± 0.16 |
| | Female | 1.40 ± 0.11 | 1.62 ± 0.14 | 1.31 ± 0.07 |
| 120 | Male | 1.80 ± 0.19 | 1.67 ± 0.08 | 1.51 ± 0.17 |
| | Female | 1.69 ± 0.18 | 1.84 ± 0.20 | 1.46 ± 0.08 |

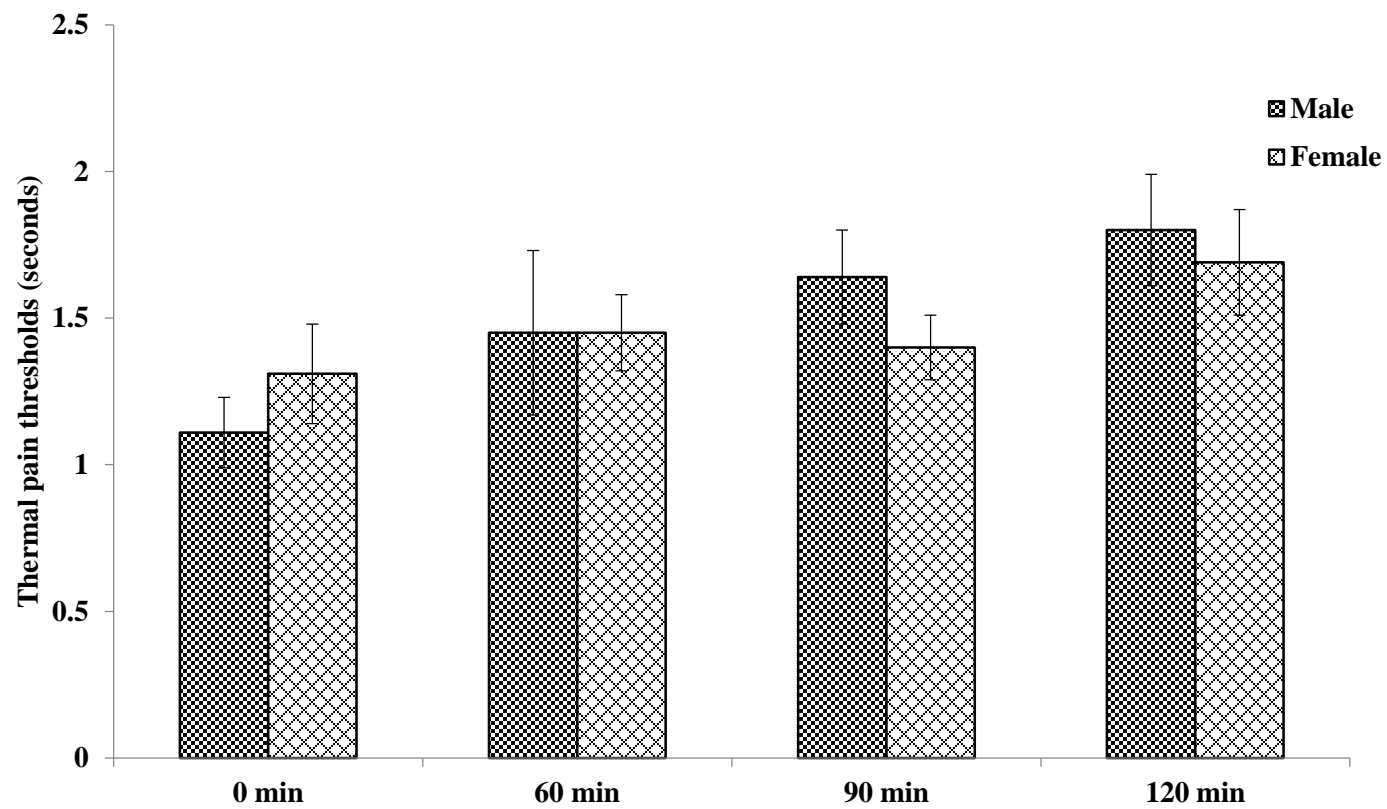


Figure 4.4: Dynamics of fluctuations in thermal pain (hot-plate) thresholds in male and female Wistar rats in control animals during the harmattan season.

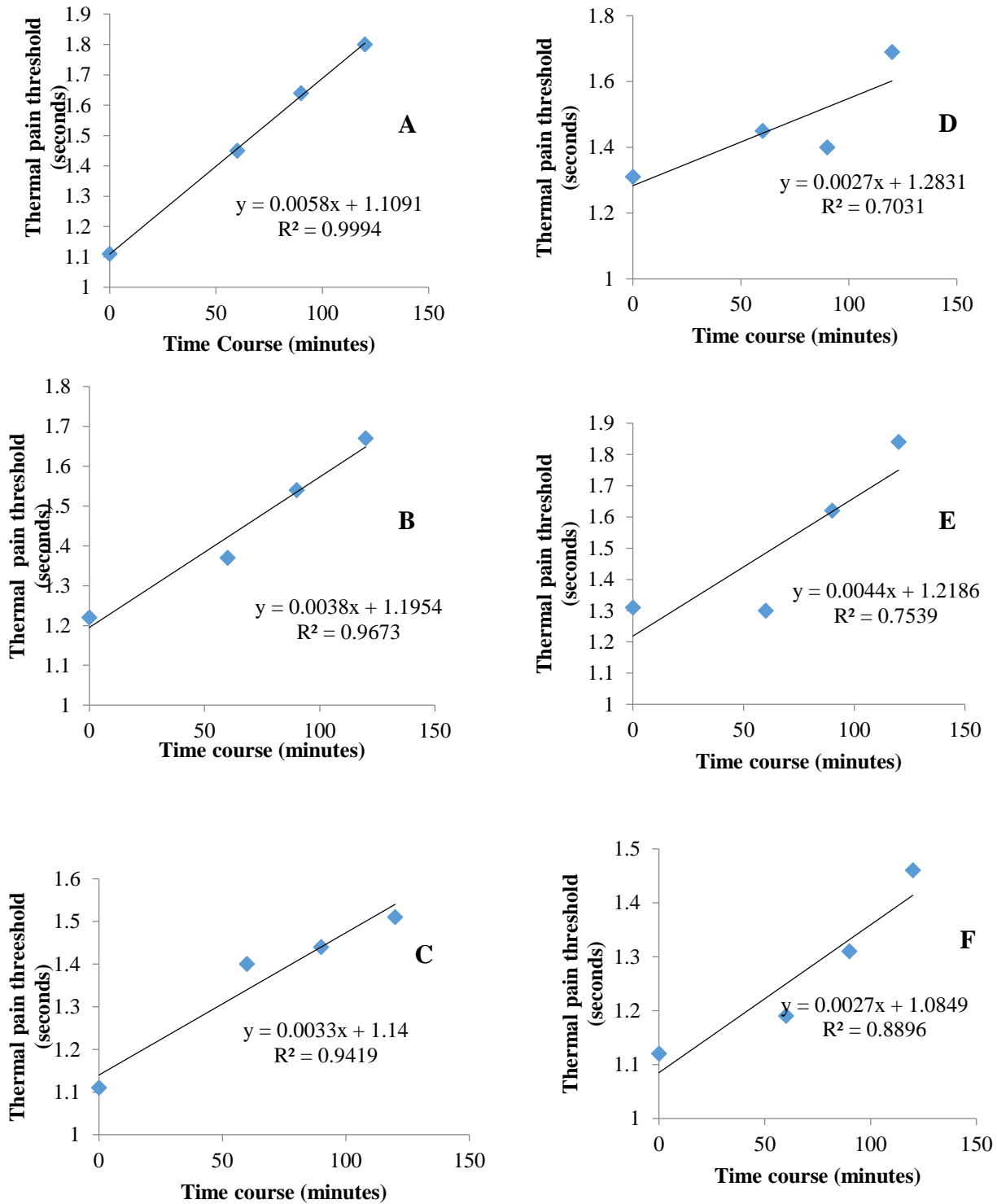


Figure 4.5: Relationship between thermal pain threshold and time course in Wistar rats during the harmattan season (n = 5). **A** = male control, **B** = male CMC, **C** = male RSV, **D** = female control, **E** = female CMC, **F**= female RSV. CMC (Carboxymethyl cellulose), RSV(Resveratrol).

Table 4.3: Relationship between of thermal pain threshold and time course in Wistar rats during the harmattan season.

| Thermal pain threshold and time course | Male | Female |
|---|-------------|---------------|
| Control | 0.898* | 0.855* |
| CMC | 0.987** | 0.730 |
| RSV | 0.940* | 0.905* |

CMC = Carboxymethyl cellulose, RSV = Resveratrol, * = (P < 0.05), ** = P < 0.001).

4.4 Nocifensive Responses to Chemical Noxious Stimulus

Results of the male and female animals subjected to chemical noxious stimulus are presented in Figure 4.6. In the early phase of the formalin test, the results were 3.4, 12 and 7 for the male control, CMC and RSV groups, respectively. CMC animals recorded a significantly ($p < 0.05$) lower pain scores compared to control or RSV group. For the female animals, the values were 8.2, 10.1 and 5.7 for control, CMC and RSV groups, respectively. There was no significant difference observed in all the female groups.

In the late phase of the formalin test, the male animals had pain scores of 4.9, 11.7 and 5.5 for control, CMC and RSV groups, respectively. The CMC group had significantly ($p < 0.05$) higher pain scores than the control or RSV group. In the female groups the pain scores were 8, 9.5 and 6.5 for control, CMC and RSV groups, respectively, and the scores were not significantly ($p > 0.05$) different. Comparison between male and female animals showed increased pain scores in female control animals in the early phase when compared to male animals in the control group ($p < 0.05$).

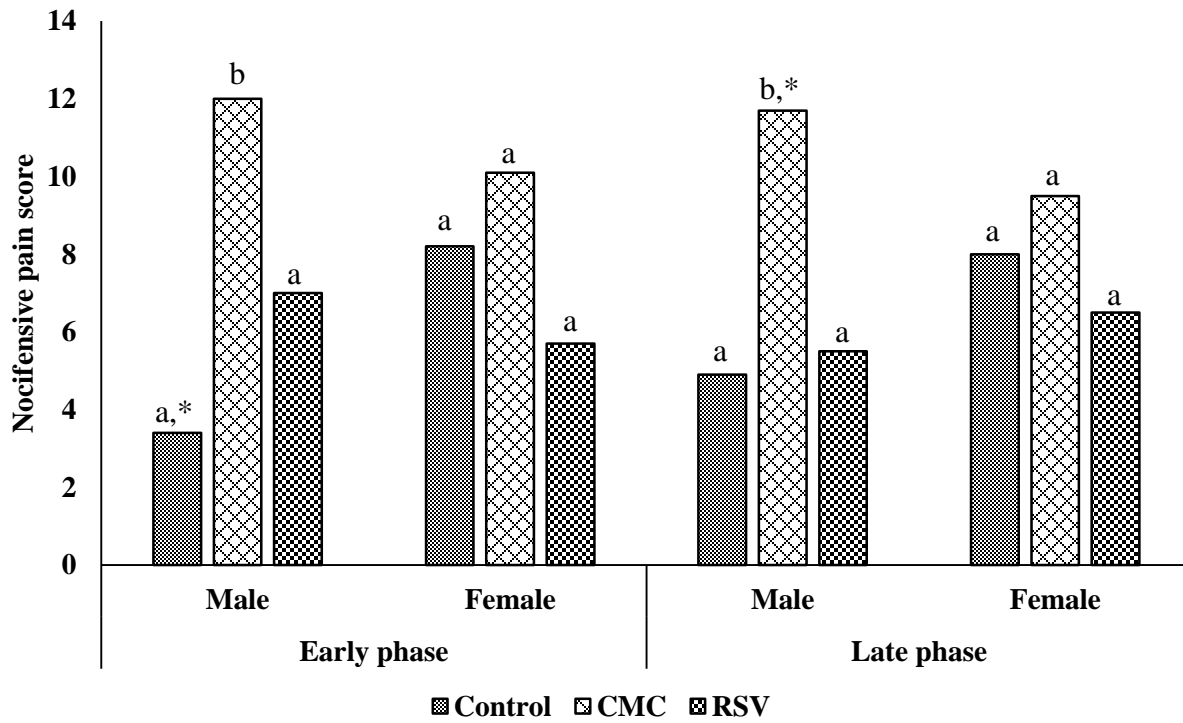


Figure 4.6: Responses to chemical pain stimulus in male and female rats ($n = 5$) during the harmattan season (Mean rank). Different superscript letters ^{a,b} indicate significant ($p < 0.05$) difference. * male vs female ($p < 0.05$). CMC = Carboxymethyl cellulose, RSV = Resveratrol.

4.5 Assessment of Electrolyte Concentrations

4.5.1 Sodium ion concentrations

4.5.1.1 Mechanical noxious stimulation

Electrolyte concentrations obtained during the study are represented in Table 4.4 - 4.6. Sodium ion concentrations (mmol/L) in male groups, exposed to mechanical noxious stimuli were 154.03 ± 0.73 , 150.47 ± 1.30 , 149.93 ± 1.99 , 152.07 ± 2.19 for control, CMC, RSV and naïve groups, respectively. The values were not significantly different between the groups ($p > 0.05$). The sodium ion concentrations (mmol/L) for the female exposed to mechanical noxious stimuli were 153.87 ± 0.43 , 153.27 ± 1.35 , 150.43 ± 1.56 , 146.53 ± 1.53 for control, CMC, RSV and naïve groups, respectively. Sodium ion concentrations obtained in the group subjected to mechanical noxious stimulus were significantly higher ($p < 0.05$) in the control and CMC groups than in the naïve group.

A Two-way ANOVA showed that only groups and not sex or groups \times sex significantly affected the sodium ion concentrations [$F(3, 16) = 3.773$, $p < 0.05$; $F(1, 16) = 0.324$, $p < 0.05$; $F(3, 16) = 2.794$; $p < 0.05$ respectively]. *Posthoc* analysis with Tukey's multiple comparisons showed significantly lower concentrations in naïve than control group. Further comparisons revealed that male naïve animals had a higher sodium ion concentration than female naïve animals ($p < 0.05$).

4.5.1.2 Chemical noxious stimulation

Sodium ion concentrations (mmol/L) in male groups exposed to chemical noxious stimuli were: 153.87 ± 0.43 , 153.27 ± 1.35 , 150.43 ± 1.56 and 152.07 ± 2.19 ; for control, CMC, RSV and naïve groups, respectively. The difference in the values were not significant. Sodium ion concentrations

(mmol/L) for female groups exposed to chemical noxious stimuli were: 148.90 ± 1.74 , 149.13 ± 1.53 , 150.23 ± 1.32 and 146.53 ± 1.53 for control, CMC, RSV and naïve groups respectively.

Two-way ANOVA showed that only sex and not groups or groups \times sex, significantly affected sodium ion concentration [$F(1, 16) = 11.76, p < 0.05$; $F(3, 16) = 0.78, p > 0.05$; $F(3, 16) = 1.24, p > 0.05$, respectively]. There was also a significant ($p > 0.05$) sex difference between the values obtained in control and naïve groups, with males having higher sodium ion concentration than females.

4.5.1.3 Thermal noxious stimulation

Sodium ion concentrations (mmol/L) for male groups exposed to thermal noxious stimuli did not differ from one another (149.40 ± 0.91 , 150.43 ± 0.43 , 148.80 ± 1.28 and 152 ± 2.18 for control, CMC, RSV and naïve groups respectively). In female groups exposed to thermal noxious stimuli, sodium concentrations were 148.20 ± 1.71 , 151.30 ± 1.63 , 150.23 ± 2.04 and 146 ± 1.53 for control, CMC, RSV and naïve groups respectively. The differences in the values were not significantly different ($p > 0.05$). Furthermore, two way ANOVA showed no significant effect of interaction between groups, sex and group \times sex in sodium ion concentration in animals subjected to thermal noxious stimuli [$F(3,16)= 0.639, p > 0.05$; $F(1, 16)= 1.00, p > 0.05$; $F(3,16)= 2.04, p > 0.05$, respectively]. Male naïve groups had significantly ($p < 0.05$) higher sodium ion concentration than female naïve groups.

4.5.1.4 Sodium ion concentrations in control rats

Sodium concentrations (mmol/L) for male control rats exposed to mechanical, chemical and thermal noxious stimuli were 154.03 ± 0.73 , 153.87 ± 0.43 and 149.40 ± 0.91 , respectively ($p > 0.05$). In the sodium ion concentrations for female control animals, the values were 153.87 ± 0.43 , 148.90 ± 1.74 and 148.20 ± 1.71 for animals exposed to mechanical, chemical and thermal noxious stimuli, respectively. Male control rats exposed to thermal noxious stimuli showed a significantly lower sodium ion concentration than the male control rats, exposed to mechanical or thermal noxious stimuli ($p < 0.05$).

Sodium concentrations (mmol/L) obtained in female control rats exposed to mechanical, chemical and thermal noxious stimuli were 153.87 ± 0.43 , 148.90 ± 1.74 and 148.20 ± 1.71 respectively, but the values were not significantly different. There was also no sex difference between the concentrations recorded in all the control groups.

Table 4.4: Sodium ion concentrations (mmol/L) in male and female Wistar rats subjected to different noxious stimulations during the harmattan season.

| Type of noxious stimulus | Groups | Males | Females |
|--------------------------|----------------|---------------|----------------------------|
| Mechanical | Control | 154.03 ± 0.73 | 153.87 ± 0.43 [#] |
| | CMC | 150.47 ± 1.30 | 153.27 ± 1.35 [#] |
| | RSV | 149.93 ± 1.99 | 150.43 ± 1.56 |
| | Naïve | 152.07 ± 2.19 | 146.53 ± 1.53 [^] |
| Thermal | Control | 149.4 ± 0.91 | 148.20 ± 1.71 |
| | CMC | 150.43 ± 0.44 | 151.30 ± 1.63 |
| | RSV | 148.80 ± 1.28 | 150.23 ± 2.04 |
| | Naïve | 152.07 ± 2.19 | 146.53 ± 0.93 [^] |
| Chemical | Control | 153.87 ± 0.43 | 148.90 ± 1.74 [^] |
| | CMC | 153.27 ± 1.35 | 149.13 ± 1.53 |
| | RSV | 150.43 ± 1.56 | 150.23 ± 1.32 [^] |
| | Naïve | 152.07 ± 2.19 | 146.53 ± 1.53 |

* $p < 0.05$ vs. control; [#] $p < 0.05$ vs. naïve. [^] $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

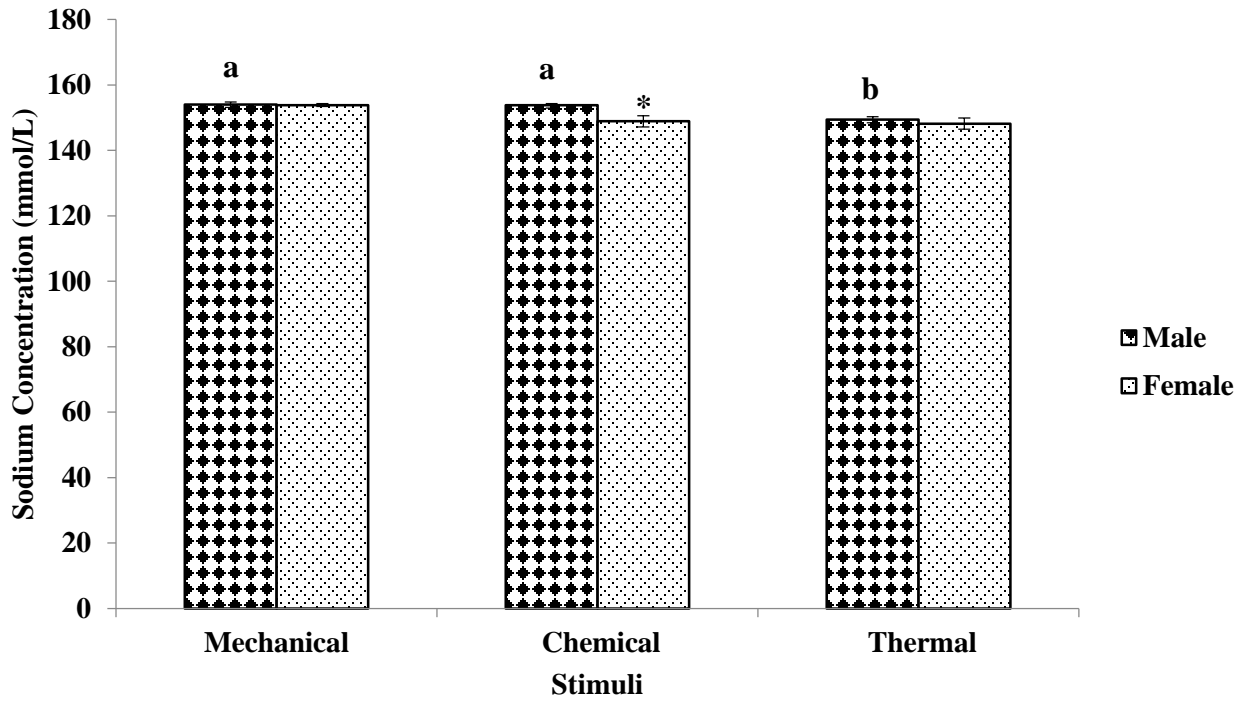


Figure 4.7: Sodium ion concentrations in control rats subjected to mechanical, chemical and thermal noxious stimuli during the harmattan season. Different superscript letters ^{a,b,c} indicate significant ($p < 0.05$) difference. * male vs female ($p < 0.05$).

4.5.2 Potassium ion concentrations

4.5.2.1 Mechanical noxious stimulation

Potassium ion concentrations (mmol/L) in male groups exposed to mechanical noxious stimuli were 1.72 ± 0.28 , 2.18 ± 0.21 , 2.03 ± 0.54 , 1.09 ± 0.16 for control, CMC, RSV and naïve groups, respectively. No significant change was observed for potassium ion concentration in the male groups. Potassium ion concentrations (mmol/L) in the female rats exposed to mechanical noxious stimuli were 2.25 ± 0.38 , 2.17 ± 0.20 , 2.32 ± 0.27 , 0.98 ± 0.12 for control, CMC, RSV and naïve groups respectively. Naïve female animals had significantly ($p < 0.05$) lower potassium ion concentration than those of the control and RSV groups. The differences in the concentrations obtained between male and female animals were not statistically significant.

4.5.2.2 Chemical noxious stimulation

Potassium ion concentrations (mmol/L) obtained in male groups exposed to chemical noxious stimulus were; 0.67 ± 0.05 , 1.23 ± 0.09 , 1.17 ± 1.33 , 1.09 ± 0.16 for control, CMC, RSV and naïve groups, respectively. Potassium ion concentration in the CMC group was higher ($p < 0.05$) than that of the control group. Potassium ion concentrations (mmol/L) recorded in female groups exposed to chemical noxious stimuli were; 1.20 ± 0.08 , 1.58 ± 0.88 , 1.22 ± 0.07 and 0.98 ± 0.12 for control, CMC, RSV and naïve groups, respectively. The naïve group had the least concentration, which was significantly lower ($p < 0.05$) than that of the CMC group. Two-way ANOVA showed that groups, sex, groups \times sex exerted significant effect on potassium ion concentration [$F(3, 16) = 6.28$, $p < 0.05$; $F(1, 16) = 6.20$, $p < 0.05$; $F(3, 16) = 3.18$, $p < 0.05$) respectively]. Male rats had significantly lower ($p < 0.05$) potassium ion concentration than their female counterparts in the control and CMC groups.

4.5.2.3 Thermal noxious stimulation

Potassium ion concentrations (mmol/L) obtained in male groups exposed to thermal noxious stimulus (1.07 ± 0.18 , 0.92 ± 0.12 , 0.75 ± 0.16 , 1.09 ± 0.16 for control, CMC, RSV and naïve groups, respectively) were not significantly different. Potassium ion concentration (mmol/L) in female groups exposed to thermal noxious stimuli were: 0.87 ± 0.11 mmol/L, 0.98 ± 0.18 , 0.97 ± 0.09 and 0.98 ± 0.12 for control, CMC, RSV and naïve groups, respectively did not differ significantly from one another ($p > 0.05$). Groups, sex and groups \times sex had no significant effect on potassium ion concentration [$F(3, 16) = 0.54$, $p > 0.05$; $F(1, 16) = 0.01$, $p < 0.05$; $F(3, 16) = 0.84$, $p < 0.05$), respectively].

4.5.2.4 Potassium ion concentrations in control rats

Potassium ion concentrations (mmol/L) in male control animals exposed to mechanical, chemical and thermal noxious stimuli were: 1.72 ± 0.28 , 0.67 ± 0.05 and 1.07 ± 0.18 respectively. There was no statistically significant difference in all the groups. The Potassium ion concentrations (mmol/L) in female control rats exposed to mechanical, chemical and thermal noxious stimuli were; 2.25 ± 0.38 , 1.20 ± 0.08 and 0.87 ± 0.11 , respectively. The Potassium ion concentrations were significantly higher ($p > 0.05$) in the controls rats exposed to mechanical noxious stimulus than those recorded for the control groups, exposed to chemical and thermal noxious stimuli; but the difference in concentrations between male and female animals was not statistically significant.

Table 4.5: Potassium ion concentrations (mmol/L) in male and female Wistar rats subjected to different noxious stimulations during the harmattan season.

| Type of noxious stimulus | Groups | Males | Females |
|--------------------------|----------------|---------------------------|--------------------------|
| Mechanical | Control | 1.72 ± 0.28 | 2.25 ± 0.38 [#] |
| | CMC | 2.18 ± 0.21 | 2.17 ± 0.20 |
| | RSV | 2.03 ± 0.54 | 2.32 ± 0.27 [#] |
| | Naïve | 1.09 ± 0.16 | 0.98 ± 0.11 |
| Thermal | Control | 1.07 ± 0.18 | 0.87 ± 0.11 |
| | CMC | 0.92 ± 0.12 | 0.98 ± 0.18 |
| | RSV | 0.75 ± 0.16 | 0.97 ± 0.09 |
| | Naïve | 1.09 ± 0.16 | 0.98 ± 0.12 |
| Chemical | Control | 0.67 ± 0.05 [^] | 1.20 ± 0.07 |
| | CMC | 1.23 ± 0.09 ^{^*} | 1.58 ± 0.16 [#] |
| | RSV | 1.17 ± 0.13 | 1.22 ± 0.07 |
| | Naïve | 1.09 ± 0.16 | 0.98 ± 0.12 [#] |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

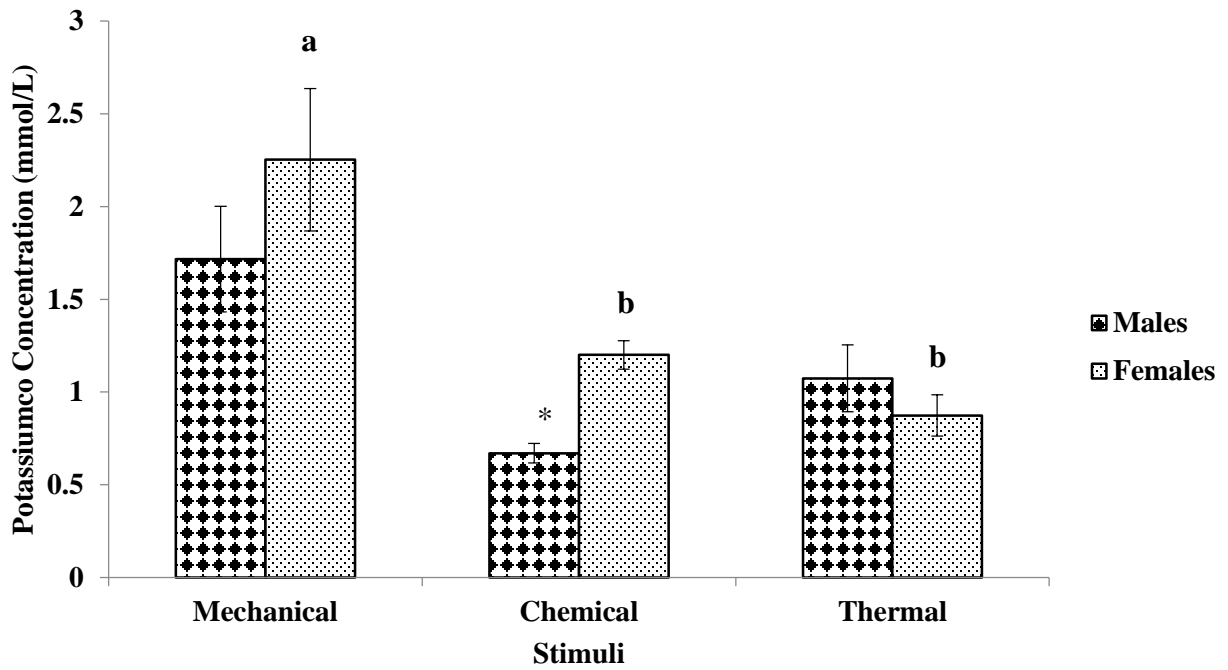


Figure 4.8: Potassium ion concentration in control rats subjected to mechanical, chemical and thermal noxious stimulation during the harmattan season. Different superscript letters ^{a,b} indicate significant difference $p < 0.05$. *= male vs. female ($p < 0.05$).

4.5.3 Chloride ion concentrations

4.5.3.1 Mechanical noxious stimulation

Chloride ion concentrations (mmol/L) obtained in male groups exposed to mechanical noxious stimulus (88.40 ± 1.67 , 91.90 ± 4.39 , 88.20 ± 2.06 , 87.27 ± 0.95 ; for control, CMC, RSV and naïve groups, respectively) did not differ significantly. There was no significant difference in chloride ion concentrations (mmol/L) in the female rats exposed to mechanical noxious stimuli (88.07 ± 1.81 , 87.7 ± 0.92 , 90.30 ± 1.22 , 91.17 ± 1.10 for control, CMC, RSV and naïve groups, respectively). Two-way ANOVA showed that groups, sex and groups \times sex had no significant effect on chloride ion concentration [$F(3, 16) = 0.20$, $p > 0.05$; $F(1, 16) = 0.06$, $p > 0.05$; $F(3, 16) = 1.45$, $p > 0.05$) respectively]. The difference between male and female chloride ion concentration was not statistically significant.

4.5.3.2 Chemical noxious stimulation

Chloride ion concentrations (mmol/L) recorded in the male groups exposed to chemical noxious stimulus were: 88.07 ± 1.81 , 87.70 ± 0.92 , 90.30 ± 1.22 , 87.27 ± 0.95 for control, CMC, RSV and naïve groups respectively, the concentrations did not significantly differ. Chloride ion concentrations (mmol/L) for female groups exposed to chemical noxious stimuli (88.47 ± 1.02 , 91.23 ± 0.59 , 88.27 ± 0.95 and 91.17 ± 1.10 for control, CMC, RSV and naïve groups, respectively), were also not significantly different. Chloride ion concentrations in male CMC group was lower than those obtained in female CMC group ($p < 0.05$). Similarly, male naïve groups also had significantly lower chloride ion concentration than female naïve group ($p < 0.05$).

4.5.3.3 Thermal noxious stimulation

Thermal noxious stimulus did not induce any significant change in chloride ion concentration (mmol/L) of male groups (89.77 ± 1.49 , 89.23 ± 1.04 , 90.50 ± 0.44 , 87.27 ± 0.95 for control, CMC, RSV and naïve groups, respectively). Chloride ion concentration (mmol/L) recorded for female rats exposed to thermal noxious stimulation (88.97 ± 0.96 , 87.30 ± 2.95 , 90.33 ± 1.07 and 91.17 ± 1.09 for control, CMC, RSV and naïve groups, respectively), were not significantly different.

Groups, sex and groups \times sex had no effects on chloride ion concentrations in the groups exposed to thermal noxious stimulation [$F(3, 16) = 0.758$, $p > 0.05$; $F(1, 16) = 0.06$, $p > 0.05$; $F(3, 16) = 1.58$, $p > 0.05$).

4.5.3.4 Chloride ion concentrations in control rats

Chloride ion concentration (mmol/L) in male control rats exposed to mechanical, chemical and thermal noxious stimulation were 88.40 ± 1.67 , 88.07 ± 1.81 and 89.77 ± 1.49 respectively, and the values did not significantly differ ($p > 0.05$). Mechanical, chemical and thermal noxious stimulation did not alter chloride ion concentrations (mmol/L) in female control rats (88.07 ± 1.81 , 88.47 ± 1.02 and 88.97 ± 0.96 , respectively).

Table 4.6: Chloride ion concentrations (mmol/L) in male and female Wistar rats subjected to different noxious stimulations during the harmattan season.

| Type of noxious stimulus | Groups | Males | Females |
|--------------------------|----------------|--------------|--------------|
| Mechanical | Control | 88.4 ± 1.67 | 88.07 ± 1.81 |
| | CMC | 91.9 ± 4.38 | 87.7 ± 0.92 |
| | RSV | 88.2 ± 2.06 | 90.3 ± 1.21 |
| | Naïve | 87.27 ± 0.95 | 91.17 ± 1.10 |
| Thermal | Control | 89.77 ± 1.49 | 88.97 ± 0.96 |
| | CMC | 89.23 ± 1.04 | 87.3 ± 2.95 |
| | RSV | 90.5 ± 0.44 | 90.33 ± 1.07 |
| | Naïve | 87.27 ± 0.95 | 91.17 ± 1.10 |
| Chemical | Control | 88.07 ± 1.81 | 88.47 ± 1.01 |
| | CMC | 87.7 ± 0.91 | 91.23 ± 0.59 |
| | RSV | 90.3 ± 1.21 | 88.27 ± 0.95 |
| | Naïve | 87.27 ± 0.95 | 91.17 ± 1.10 |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

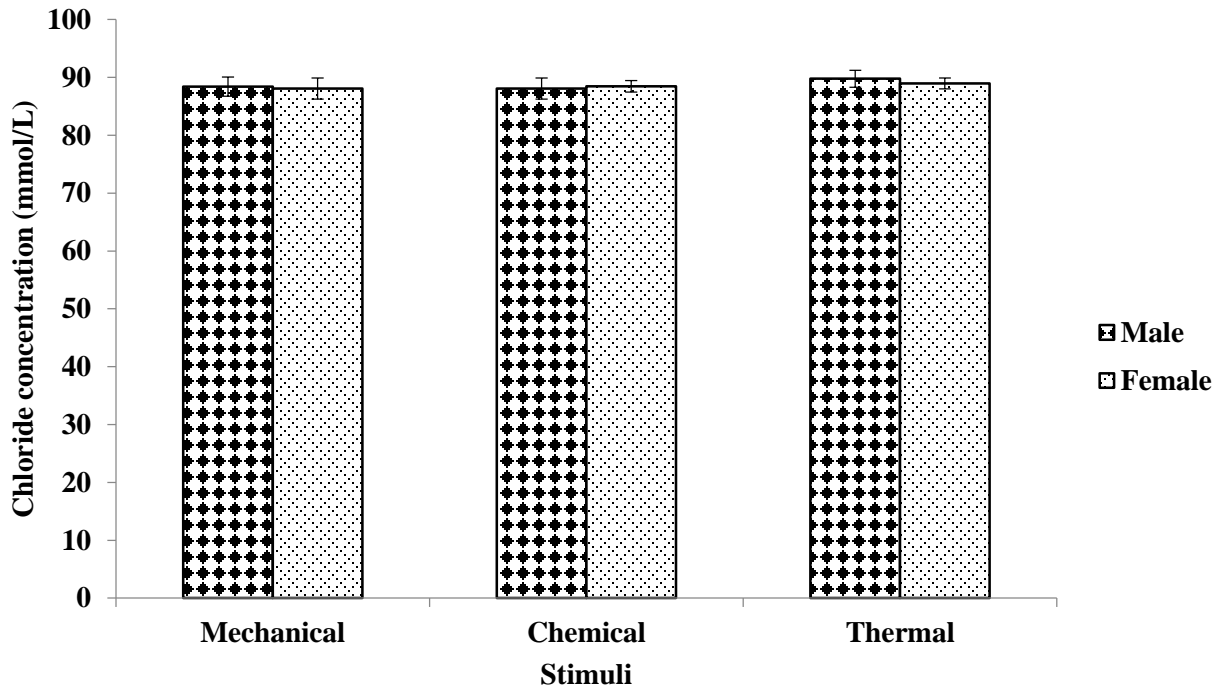


Figure 4.9: Chloride ion concentrations in control rats subjected to mechanical, chemical and thermal noxious stimulations during the harmattan season.

4.6 Erythrocyte Osmotic Fragility

4.6.1 Mechanical noxious stimulation

Percentage haemolysis of erythrocyte osmotic fragility of male and female's rats exposed to mechanical noxious stimulus for control, CMC, RSV and naïve groups are presented in Figures 4.10 - 4.16. No haemolysis was observed at 0.85% NaCl concentration.

Percentage haemolysis of male animals exposed to mechanical noxious stimulus for control, CMC, RSV and naïve groups are represented in Figure 4.10. At 0.70% NaCl concentration, CMC group had a significant ($p < 0.05$) increase in percentage haemolysis when compared to naïve animals. At 0.70% NaCl concentration for female rats, naïve animals had significantly ($p < 0.05$) lower percentage haemolysis, when compared to those of control and CMC groups. Only Groups significantly affected percentage haemolysis at 0.70% NaCl concentration [$F(3, 16) = 16.49, p < 0.001$]. There was no significant difference in percentage haemolysis between male and female animals.

At 0.60% NaCl concentration, percentage haemolysis in CMC group was significantly ($p < 0.05$) higher than in control and naïve groups. Female control and CMC rats at 0.60% NaCl concentration groups had significantly ($p < 0.05$) higher percentage haemolysis, when compared to RSV and naïve groups. Groups significantly affected percentage haemolysis at 0.60% NaCl concentration [$F(3, 16) = 23.37, p < 0.001$], but no significant difference was obtained in percentage haemolysis between male and female rats.

At 0.50% NaCl concentration, RSV and CMC male rats, had a significantly ($p < 0.05$) higher percentage haemolysis when compared to both control and naïve groups, respectively. At 0.05%

NaCl concentration, female, RSV and naïve groups had significantly ($p < 0.05$) lower percentage haemolysis, when compared to control or the CMC group. Groups and groups \times sex had a significant effect on percentage haemolysis at 0.50% NaCl concentration [$F(3, 16) = 24.65, p < 0.001$; $F(3, 16) = 11.75, p < 0.001$]. Sex did not affect percentage haemolysis at 0.50% NaCl concentration [$F(1, 16) = 0.72, p > 0.05$].

At 0.40% NaCl concentration, RSV and CMC treated rats also had higher percentage haemolysis than naïve group ($p < 0.05$), but not the control group ($p > 0.05$). At 0.40% NaCl concentration, female RSV and naïve groups had significantly ($p < 0.05$) lower percentage haemolysis, when compared to control and CMC groups. Groups and groups \times sex significantly affected percentage haemolysis at 0.40% NaCl concentration [$F(3, 16) = 11.59, p < 0.001$; $F(3, 16) = 9.192, p < 0.001$]. Sex did not alter the percentage haemolysis at 0.40% NaCl concentration [$F(1, 16) = 1.70, p > 0.05$].

At 0.30% NaCl concentration percentage haemolysis recorded for RSV and CMC was higher ($p < 0.05$) than that recorded in control or naïve group. At 0.03% NaCl concentration, female RSV and naïve groups had significantly ($p < 0.05$) lower percentage haemolysis, when compared to control and CMC groups, respectively. Complete haemolysis was recorded for all the male and female groups at 0.20% NaCl concentration. Groups and groups \times sex significantly affected percentage haemolysis at 0.30% NaCl concentration [$F(3, 16) = 28.33, p < 0.001$; $F(3, 16) = 12.93, p < 0.001$]. Sex alone did not affect percentage haemolysis at 0.30% NaCl concentration [$F(1, 16) = 0.02, p > 0.05$].

4.6.2 Chemical noxious stimulation

In male rats exposed to chemical noxious stimulation, percentage haemolysis was significantly ($p < 0.05$) lower for naïve when compared to those of control and CMC groups at 0.70% NaCl concentration. Percentage haemolysis recorded in female rats exposed to chemical noxious stimulation for control, CMC, RSV and naïve group at 0.70% NaCl concentration did not differ. At 0.70% NaCl concentration, percentage haemolysis significantly differed among the various groups. [$F(3, 16) = 9.55, p < 0.05$]. Sex and groups \times sex did not affect percentage haemolysis at 0.70% NaCl concentration [$F(1, 16) = 1.18, p > 0.05$]; $F(3, 16) = 0.55; p > 0.05$].

At 0.60% NaCl concentration, only male naïve group had a significantly ($p < 0.05$) lower percentage haemolysis than CMC or control group. The percentage haemolysis at 0.60% NaCl concentration differed significantly among the groups [$F(3, 16) = 6.89, p < 0.05$], but sex and groups \times sex did not alter the percentage haemolysis at 0.60% NaCl concentration [$F(1, 16) = 0.66, p > 0.05$]; $F(3, 16) = 0.34; p > 0.05$].

No significant ($p > 0.05$) difference was observed in percentage haemolysis in all the male groups at 0.50%, 0.40%, and 0.30% NaCl concentrations. Similarly, at 0.50% and 0.30% NaCl concentration in female animals, there was also no significant ($p > 0.05$) difference in percentage haemolysis among the groups.

The percentage haemolysis in female rats exposed to chemical noxious stimulation recorded at 0.40% NaCl concentration in naïve and RSV groups was significantly ($p < 0.05$) lower, when compared to controls. Two-way ANOVA showed that the percentage haemolysis differed among the groups [$F(1, 16) = 5.50, p < 0.05$; $F(3, 16) = 3.37, p < 0.05$] at 0.50% and 0.30% NaCl

concentration, respectively. At 0.40% NaCl concentration, the differences in percentage haemolysis among the male and female rats were not significant.

4.6.3 Thermal noxious stimulation

At 0.70% and 0.60% NaCl concentration, RSV significantly ($p < 0.05$) reduced percentage haemolysis in male rats when compared to control and naïve groups. Percentage haemolysis in CMC female rats exposed to thermal noxious stimulation at 0.70% and 0.60% NaCl concentrations, had a significantly ($p < 0.05$) higher percentage haemolysis when compared to the control group.

Two-way ANOVA revealed that at 0.70% NaCl concentration groups, sex and group \times sex exerted significant effects on percentage haemolysis [$F(3,16) = 6.13, p < 0.05$; $F(1, 16) = 4.11, p < 0.05$; $F(3, 16) = 11.09, p < 0.05$) respectively]. Similarly at 0.60% NaCl concentration groups, sex and group \times sex had significant effects on percentage haemolysis [$F(3,16) = 4.29, p < 0.05$; $F(1, 16) = 7.31, p < 0.05$; $F(3, 16) = 6.46, p < 0.05$), respectively]. At 0.5%, 0.40% and 0.30% NaCl concentration for male and female animals there was no significant difference among all the groups ($p > 0.05$).

4.6.4 Percentage haemolysis in control rats

Percentage haemolysis in male control rats exposed to mechanical, chemical and thermal noxious stimulations did not significantly differ ($p > 0.05$) in all the concentrations of NaCl. Percentage haemolysis of female control rats subjected to mechanical, chemical and thermal noxious stimulation are represented in Figure 4.16. At 0.70% NaCl concentration, control group exposed to thermal noxious stimulation had significantly ($p < 0.05$) lower percentage haemolysis than

control rats exposed to mechanical and chemical noxious stimulation. At 0.60% NaCl concentration, percentage haemolysis of thermal control rats was significantly lower than mechanical control ($p < 0.05$). At 0.50% and 0.40% NaCl concentration mechanical control had a significantly higher percentage haemolysis than thermal and chemical control groups ($p < 0.05$). At 0.05% NaCl concentration, percentage haemolysis of control rats subjected to mechanical noxious stimulus was significantly higher than controls subjected to chemical and thermal noxious stimuli ($p < 0.05$).

Percentage haemolysis was significantly different ($p < 0.05$) at 0.50% and 0.30% NaCl concentration for mechanical control male and female rats, ($56.67 \pm 4.18\%$ and $77.00 \pm 4.58\%$; $87.67 \pm 2.72\%$ and $99.33 \pm 0.67\%$, respectively). Percentage haemolysis was significantly ($p < 0.05$) lower in female than male at 0.70% NaCl concentration for control animals exposed to thermal noxious stimulation ($31.33 \pm 0.88\%$ and $22.00 \pm 1.73\%$). There was no statistically significant difference ($p > 0.05$) in percentage haemolysis between male and female control rats exposed to chemical noxious stimulation.

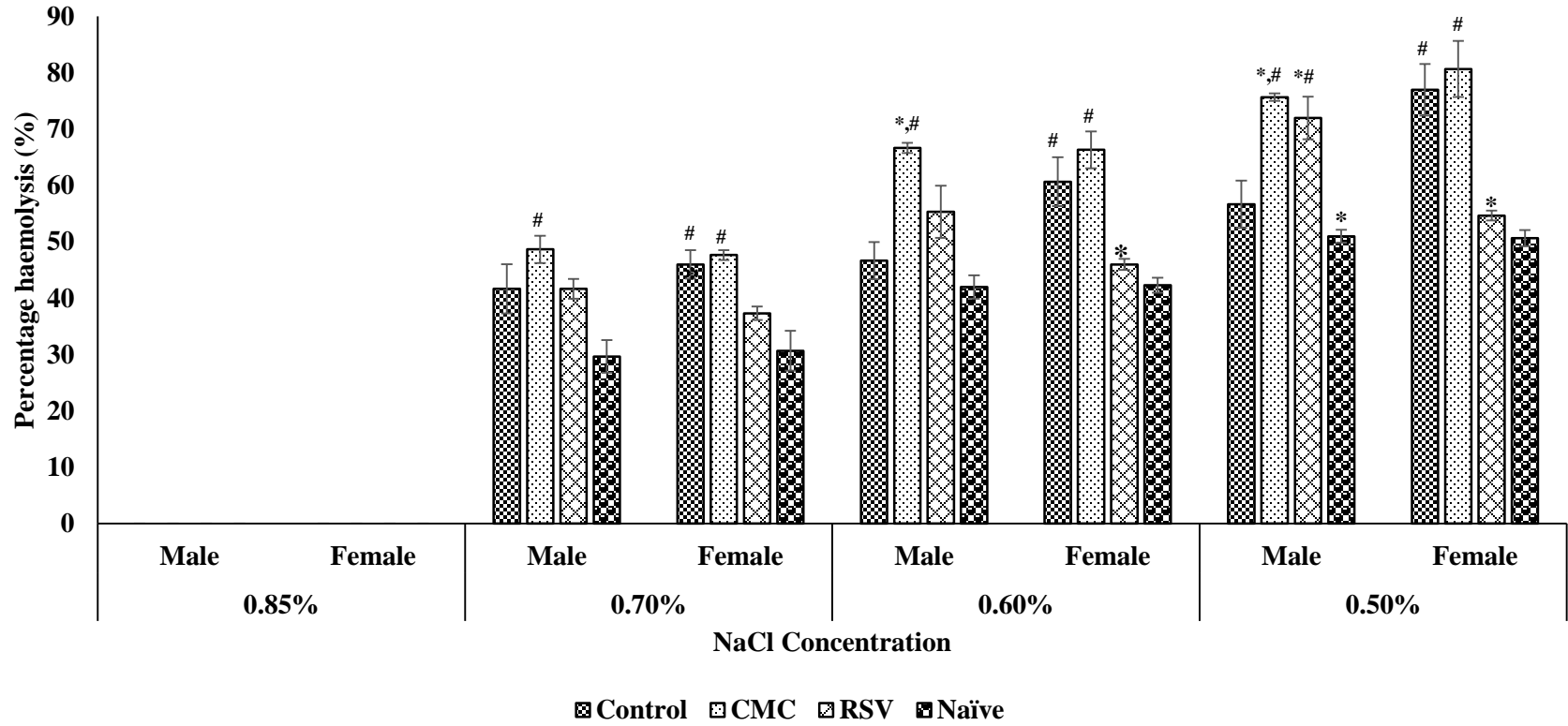


Figure 4.10: Percentage haemolysis (0.5% - 0.85% NaCl) in rats exposed to mechanical noxious stimulation during the harmattan season. * $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female, CMC = Carboxymethyl cellulose, RSV = Resveratrol.

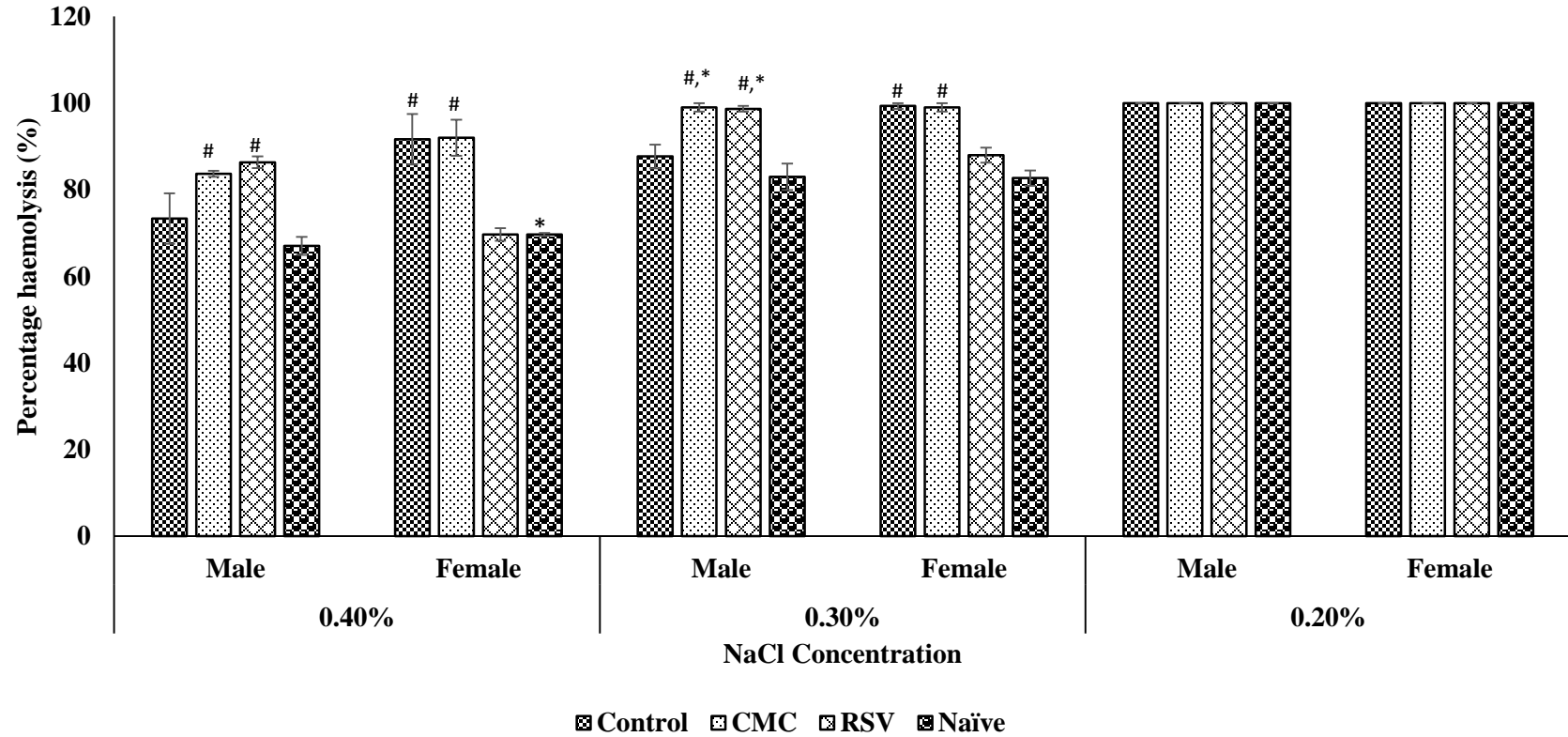


Figure 4.11: Percentage haemolysis (0.4% - 0.2% NaCl) in rats exposed to mechanical noxious stimulation during the harmattan season. * $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female, CMC = Carboxymethyl cellulose, RSV = Resveratrol.

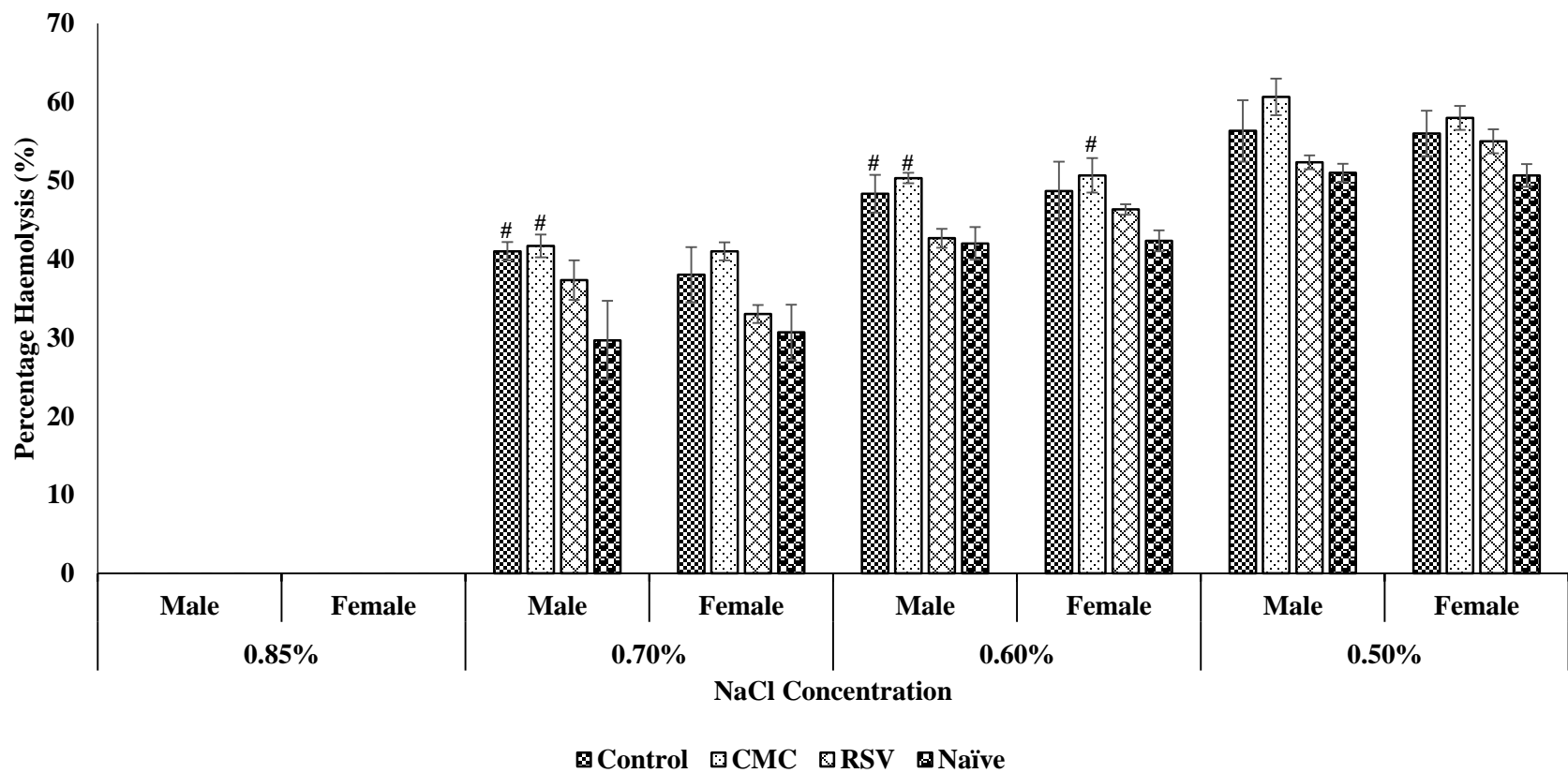


Figure 4.12: Percentage haemolysis (0.5% - 0.85% NaCl) in rats exposed to chemical noxious stimulation during the harmattan season.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve; ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

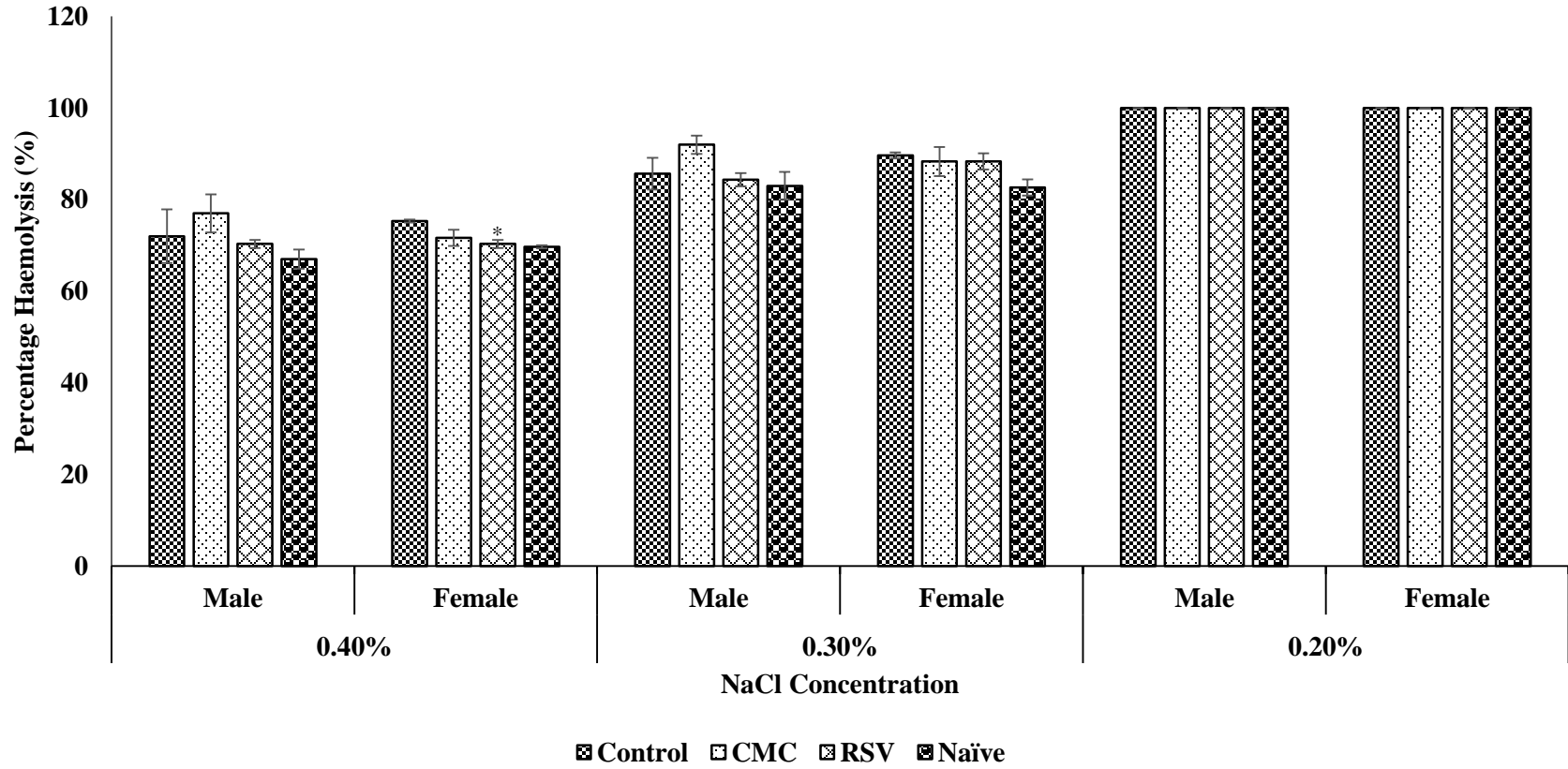


Figure 4.13: Percentage haemolysis (0.4% - 0.2% NaCl) in rats exposed to chemical noxious stimulation during the harmattan season.

* $p < 0.05$ vs. control. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

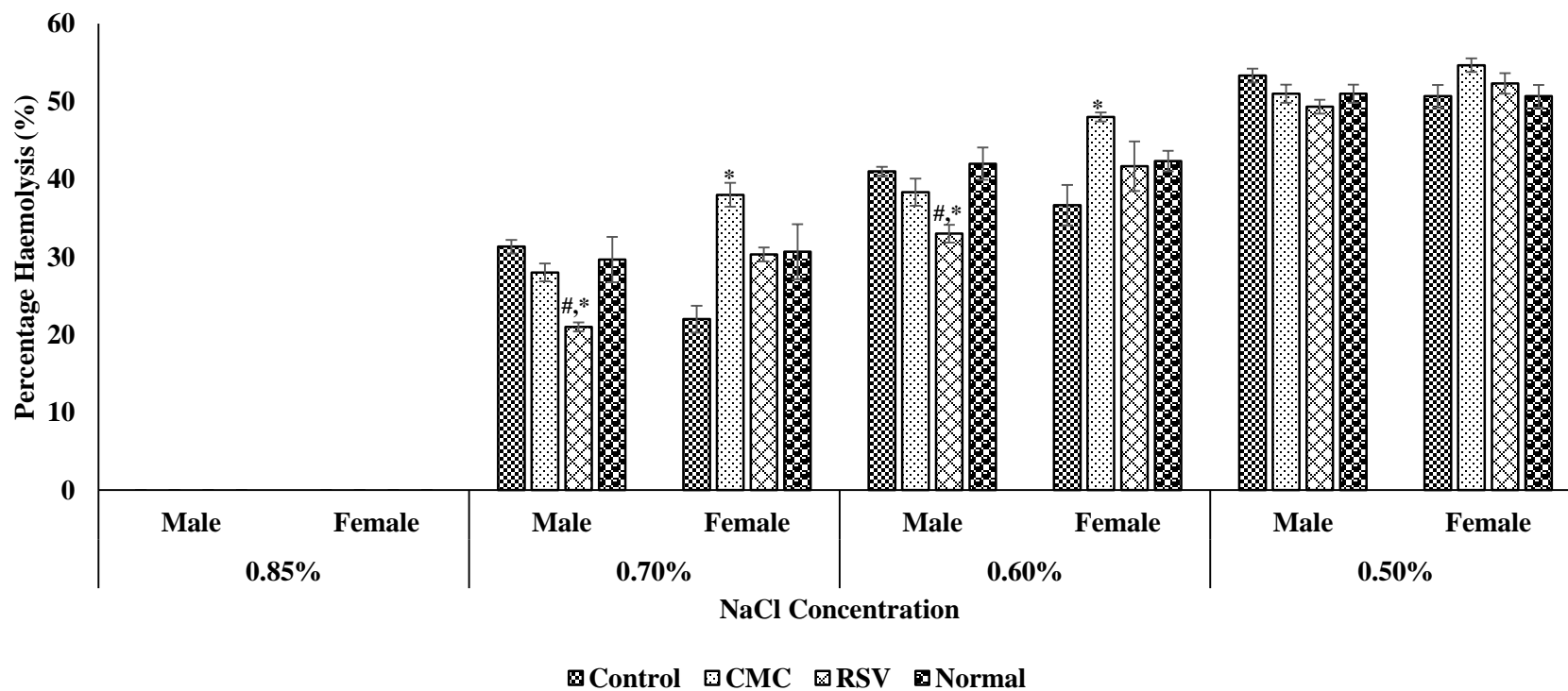


Figure 4.14: Percentage haemolysis (0.85% - 0.5% NaCl) in rats exposed to thermal noxious stimulation during the harmattan season.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

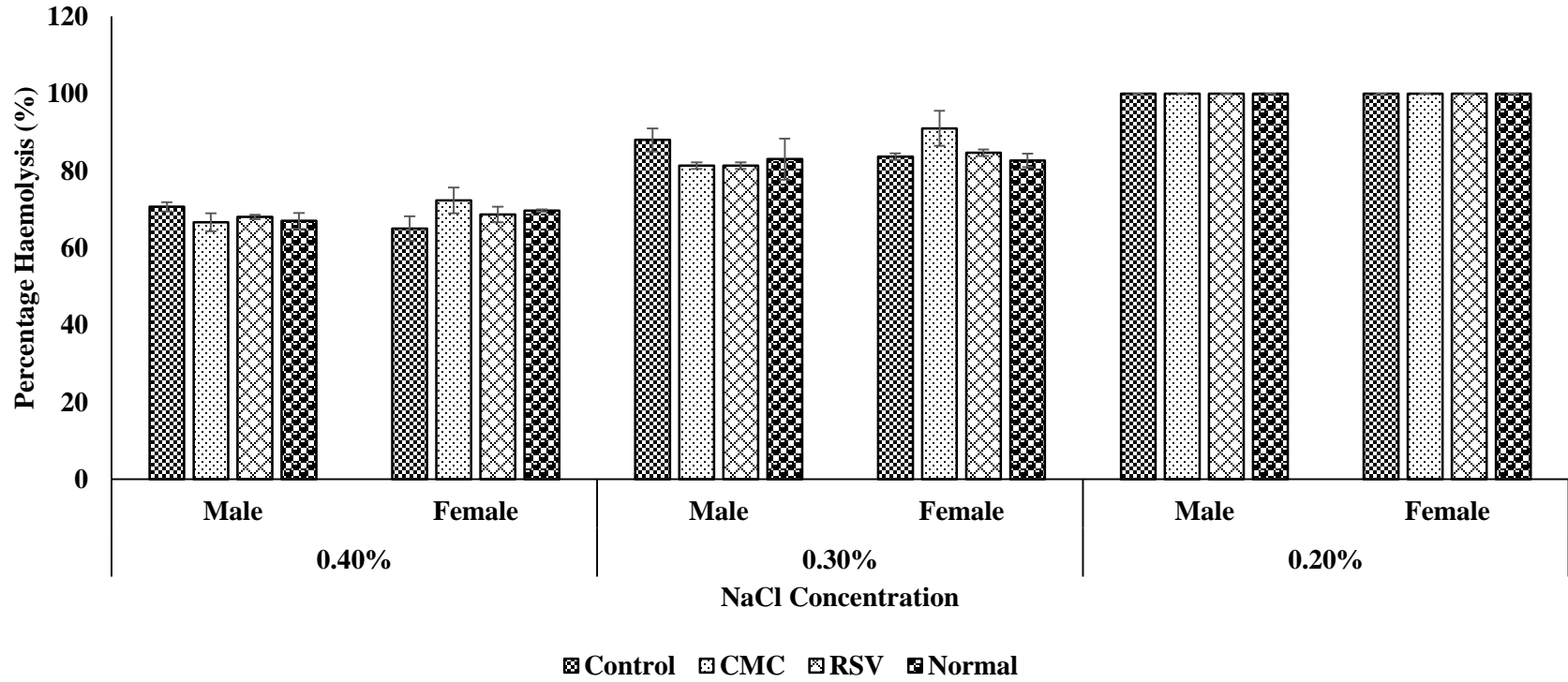


Figure 4.15: Percentage haemolysis (0.4% - 0.2% NaCl) in rats exposed to thermal noxious stimulation during the harmattan season.

CMC = Carboxymethyl cellulose, RSV = Resveratrol.

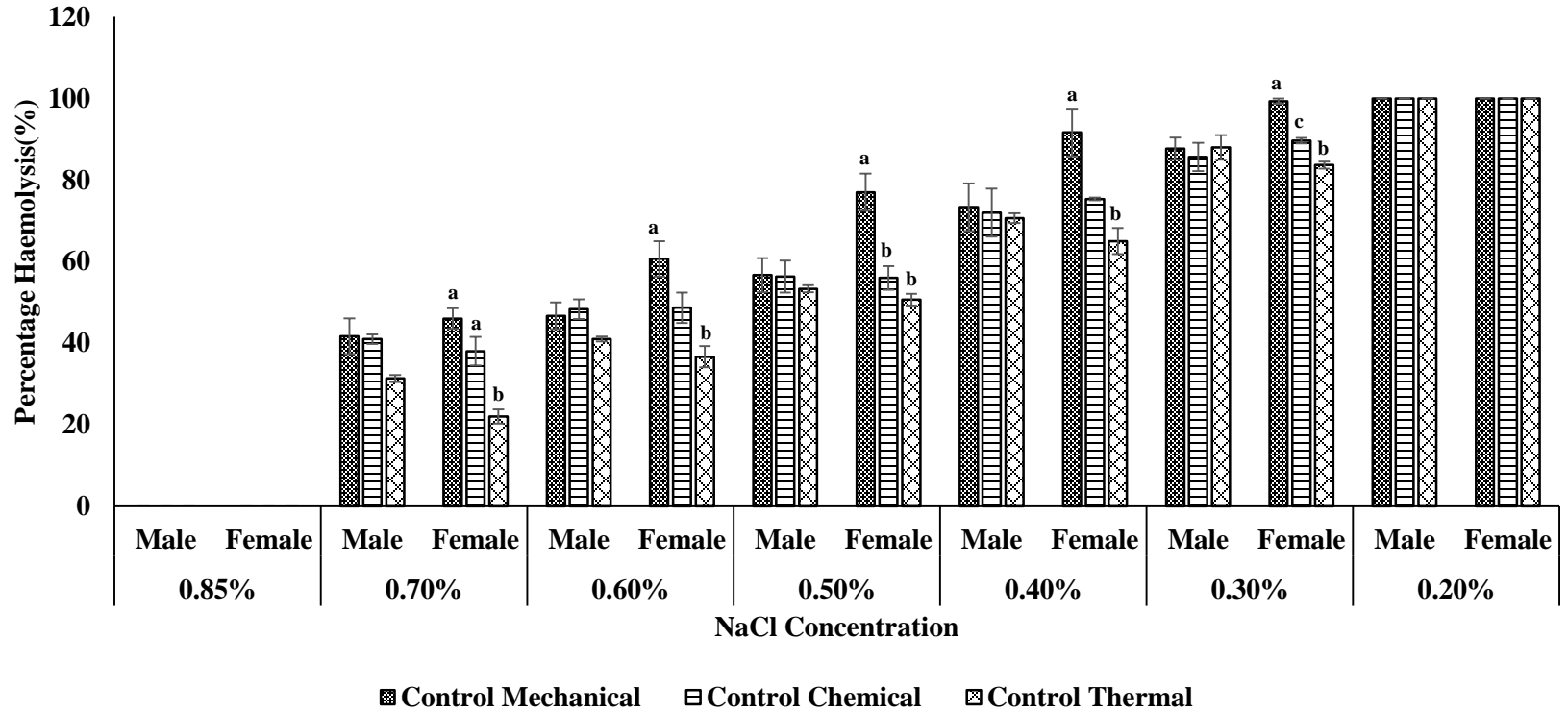


Figure 4.16: Percentage haemolysis in control rats exposed to mechanical, chemical and thermal noxious stimuli during the harmattan season. Different superscript letters ^{a,b,c} indicate significant difference $p < 0.05$; * male vs. female ($p < 0.05$).

4.7 Oxidative Stress Assessment

4.7.1 Malondialdehyde (MDA) concentration

4.7.1.1 Mechanical noxious stimulation

Malondialdehyde (MDA) concentration (nmol/ml) obtained in male control, CMC, RSV and naïve rats exposed to mechanical noxious stimulation are presented in Figures 4.17 - 4.20 (47.33 ± 1.45 , 47.67 ± 1.86 , 41.00 ± 0.58 and 42.00 ± 1.15 , respectively). RSV group had the lowest ($p < 0.05$) MDA concentration when compared to the control group. Malondialdehyde concentrations (nmol/ml) in female animals subjected to mechanical noxious stimulation were 48.33 ± 1.76 , 46.67 ± 1.45 , 47.67 ± 2.19 and 40.00 ± 0.58 for control, CMC, RSV and naïve groups, respectively. The concentrations in control and RSV groups were significantly ($p < 0.05$) higher than in the naïve group. Groups and group \times sex exerted significant effects on MDA concentration [$F(3, 16) = 8.85$, $p < 0.05$; $F(3, 16) = 12.56$, $p < 0.05$], respectively]. In the RSV groups there was a significant sex difference with male having a lower MDA concentration than the female rats ($p < 0.05$).

4.7.1.2 Chemical noxious stimulation

Malondialdehyde (MDA) concentrations (nmol/ml) in male animals subjected to chemical noxious stimulation were 47.33 ± 1.20 , 50.67 ± 0.67 , 47.00 ± 1.54 and 42.00 ± 1.15 for control, CMC, RSV and naïve groups, respectively. The concentration was lowest ($p < 0.05$), in naïve group compared to control, CMC, and RSV groups. Malondialdehyde concentrations in female animals exposed to chemical noxious stimuli for control, CMC and RSV and naïve groups were: 42.67 ± 2.19 , 46.00 ± 1.15 , 45.67 ± 1.33 and 40.00 ± 0.58 , respectively. There was no significant difference in malondialdehyde concentration,

recorded in all the groups although the naïve group had the lowest concentration. Groups and sex had significant effects on MDA concentration [$F(3, 16) = 12.01, p < 0.05$; $F(1, 16) = 3.42, p < 0.05$) respectively]. There was a significant sex difference between control and CMC groups, with male having a lower MDA concentration than the female animals ($p < 0.05$) in both groups.

4.7.1.3 Thermal noxious stimulation

Malondialdehyde (MDA) concentrations (nmol/ml) recorded in male animals subjected to thermal noxious stimulation were: 41.33 ± 0.67 , 40.00 ± 2.08 , 42.00 ± 1.52 and 42.00 ± 1.15 for control, CMC, RSV and naïve groups, respectively. These values were not statistically significantly different between the groups. Malondialdehyde concentrations (nmol/ml) for female rats were 43.33 ± 1.86 , 42.67 ± 0.88 , 46.33 ± 3.18 , and 40.00 ± 0.58 for control, CMC, RSV and naïve groups, respectively. Similarly, no significant difference was observed in malondialdehyde concentrations of female animals subjected to thermal noxious stimulation. Groups, sex and group \times sex had no significant effects on MDA concentration [$F(3, 16) = 1.40, p > 0.05$; $F(1, 16) = 2.12, p > 0.05$; $F(3, 16) = 1.25, p > 0.05$ respectively]. In the RSV group only, MDA concentration was lower ($p < 0.05$) in the males than female animals.

4.7.1.4 Malondialdehyde concentration in control rats

The concentrations of Malondialdehyde (MDA) concentrations (nmol/ml) in male control rats exposed to mechanical, chemical and thermal noxious stimulation were 47.33 ± 1.45 , 47.33 ± 1.20 and 41.33 ± 0.67 , respectively. MDA concentrations recorded in control rats

subjected to thermal noxious stimulation was significantly lower ($p < 0.05$) than values obtained in controls subjected to mechanical and chemical stimulations. MDA concentration in (nmol/ml) in female control rats exposed to mechanical, chemical and thermal noxious stimulation were: 48.33 ± 1.76 , 42.67 ± 2.19 and 43.33 ± 1.86 , respectively. The MDA concentrations among the female control groups did not significantly differ ($p > 0.05$).

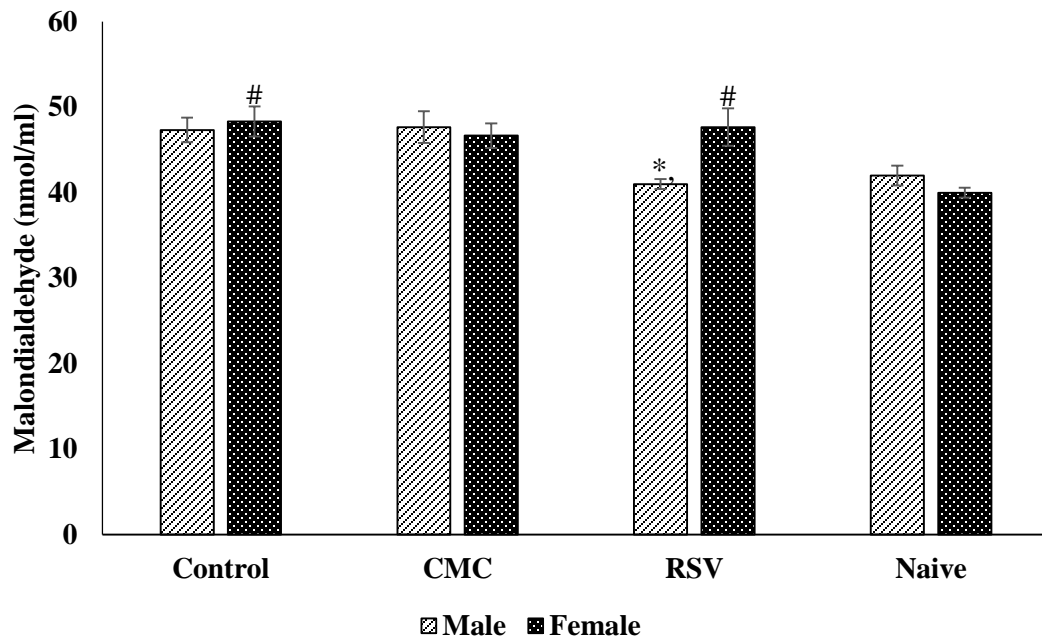


Figure 4.17: Malondialdehyde concentrations of male and female animals subjected to mechanical noxious stimulation. * $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

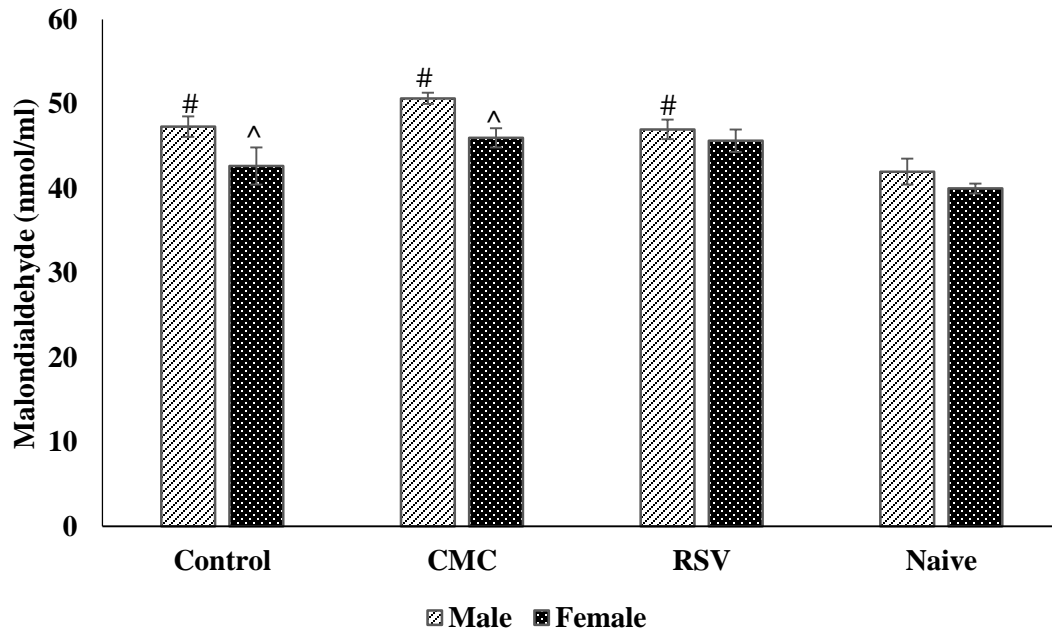


Figure 4.18: Malondialdehyde concentrations of male and female animals subjected to chemical noxious stimulation. # $p < 0.05$ vs. naïve; ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

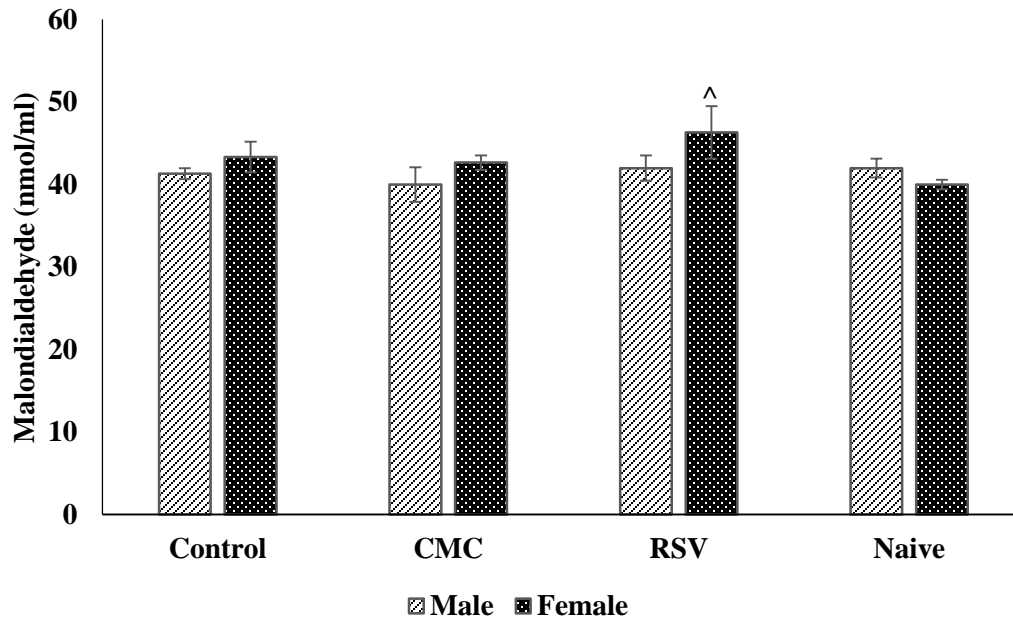


Figure 4.19: Malondialdehyde concentrations in male and female animals subjected to thermal noxious stimulation. [^] $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

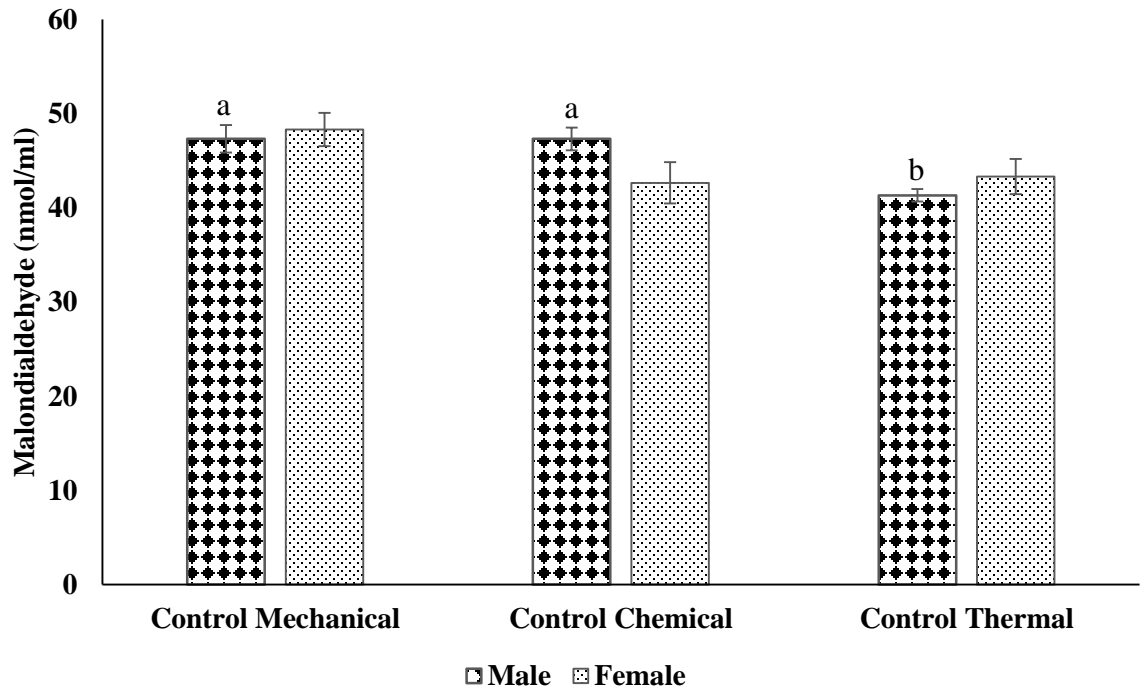


Figure 4.20: Malondialdehyde concentrations in control rats subjected to mechanical, chemical and thermal noxious stimulations during the harmattan season. Different superscript letters ^{a,b} indicate significant difference ($p < 0.05$).

4.7.2 Superoxide dismutase (SOD) concentration

4.7.2.1 Mechanical noxious stimulation

Serum concentration of superoxide dismutase (ng/ml) in male rats subjected to mechanical noxious stimulus (1.20 ± 0.10 , 1.33 ± 0.03 , 1.20 ± 0.06 and 1.43 ± 0.18 for control, CMC, RSV and naïve groups, respectively) were not significantly different between the groups. Concentrations of superoxide dismutase (ng/ml) in female animals subjected to mechanical noxious stimuli were 1.43 ± 0.07 , 1.50 ± 0.06 , 1.57 ± 0.15 and 1.13 ± 0.06 for control, CMC, RSV and naïve groups, respectively. Serum superoxide dismutase did not significantly differ among the groups. Groups \times sex interaction affected SOD activity [$F(3, 16) = 4.09$, $P < 0.05$]. Concentration of superoxide dismutase in male RSV group was significantly ($P < 0.05$) lower than that in the female RSV groups.

4.7.2.2 Chemical noxious stimulation

Superoxide dismutase concentration (ng/ml) in male rats subjected to chemical noxious stimulus were: 1.10 ± 0.00 , 1.13 ± 0.07 , 1.07 ± 0.07 and 1.43 ± 0.18 for control, CMC, RSV and naïve groups, respectively. The values were not significantly different. Superoxide dismutase concentration (ng/ml) in female rats subjected to chemical noxious stimulus (1.37 ± 0.07 , 1.27 ± 0.09 , 1.57 ± 0.24 and 1.13 ± 0.09 for control, CMC, RSV and naïve groups, respectively) did not significantly differ in all the groups. Groups \times sex interaction affected superoxide dismutase concentration [$F(3, 16) = 3.84$, $P < 0.05$]. The activity of superoxide dismutase in male RSV group was significantly ($P < 0.05$) lower than in the female RSV groups.

4.7.2.3 Thermal noxious stimulation

Superoxide dismutase concentration (ng/ml) were: 1.13 ± 0.03 , 1.37 ± 0.03 , 1.27 ± 0.09 and 1.43 ± 0.18 for control, CMC, RSV and naïve groups, respectively in male animals subjected to thermal noxious stimuli were not significantly different among the groups. Superoxide dismutase concentration (ng/ml) recorded in female animals subjected to thermal noxious stimulus were: 1.60 ± 0.06 , 1.53 ± 0.03 , 1.90 ± 0.06 and 1.13 ± 0.09 for control, CMC, RSV and naïve groups, respectively. Superoxide dismutase concentration was significantly ($p < 0.05$) higher in the control, CMC and RSV, when compared to that of the naïve group.

Groups, sex and groups \times sex interaction affected superoxide dismutase concentration [$F(3, 16) = 4.62$, $p < 0.05$; $F(1, 16) = 16.49$, $p < 0.05$; $F(3, 16) = 11.84$, $p < 0.05$], respectively]. Superoxide dismutase activities in male RSV and control groups were significantly ($p < 0.05$) lower than in female RSV or control groups, while male naïve group had significantly ($p < 0.05$) higher superoxide dismutase concentration than female naïve group.

4.7.2.4 Superoxide dismutase concentration in control rats

Superoxide dismutase concentration (ng/ml) in male control animals exposed to mechanical, chemical and thermal noxious stimuli were: 1.20 ± 0.10 , 1.10 ± 0.00 and 1.13 ± 0.03 , respectively. These values were not significantly ($p > 0.05$) different among the female control groups. The superoxide dismutase concentrations (ng/ml) in male control animals, exposed to mechanical, chemical and thermal noxious stimulation were: 1.43 ± 0.07 , $1.37 \pm$

0.07 and 1.60 ± 0.06 , respectively. There was no statistically significant ($p > 0.05$) difference in superoxide dismutase concentration among the female control groups.

Sex difference in superoxide dismutase concentration was observed with male controls having lower concentrations than their female counterparts, exposed to mechanical, chemical and thermal noxious stimulations.

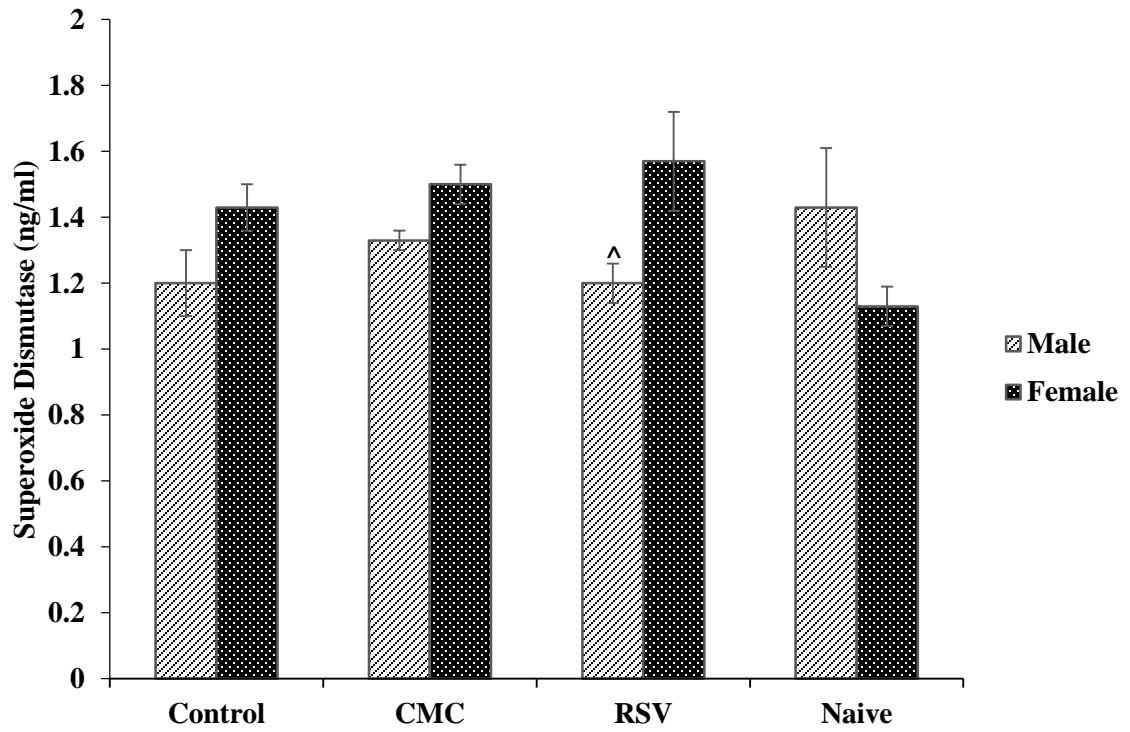


Figure 4.21: Superoxide dismutase concentration in male and female rats subjected to mechanical noxious stimulus. [^] $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

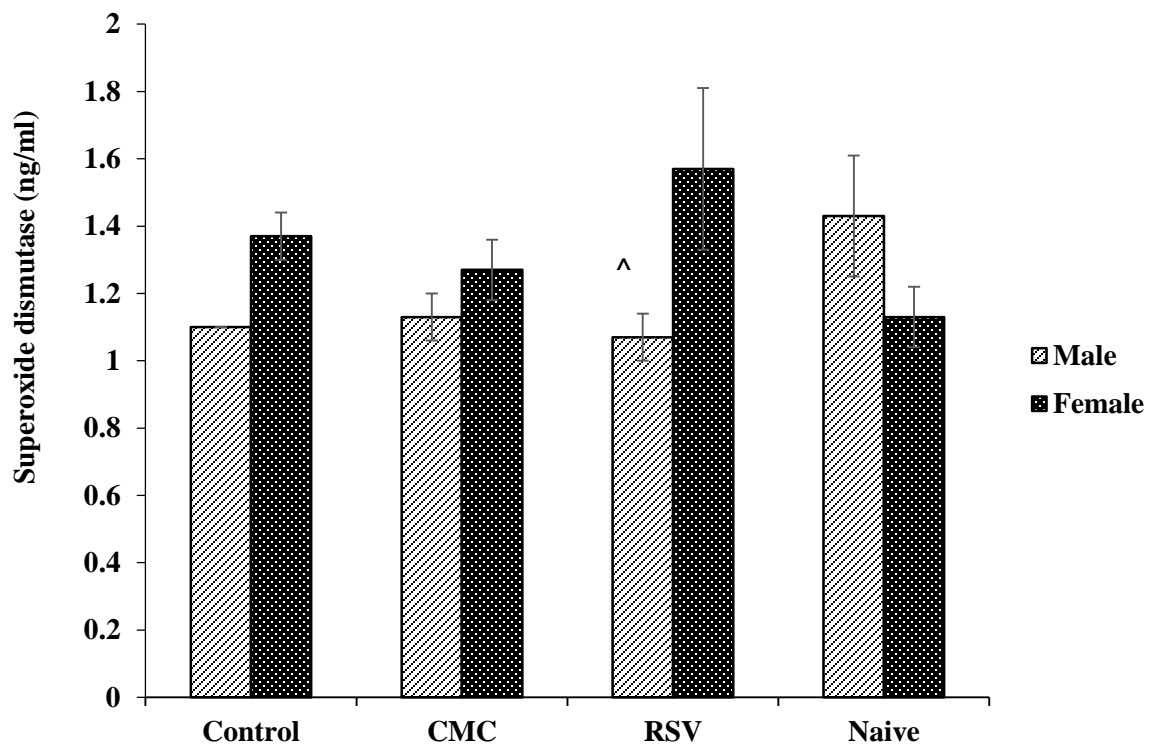


Figure 4.22: Superoxide dismutase concentration in male and female rats subjected to chemical noxious stimulus. [^] $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

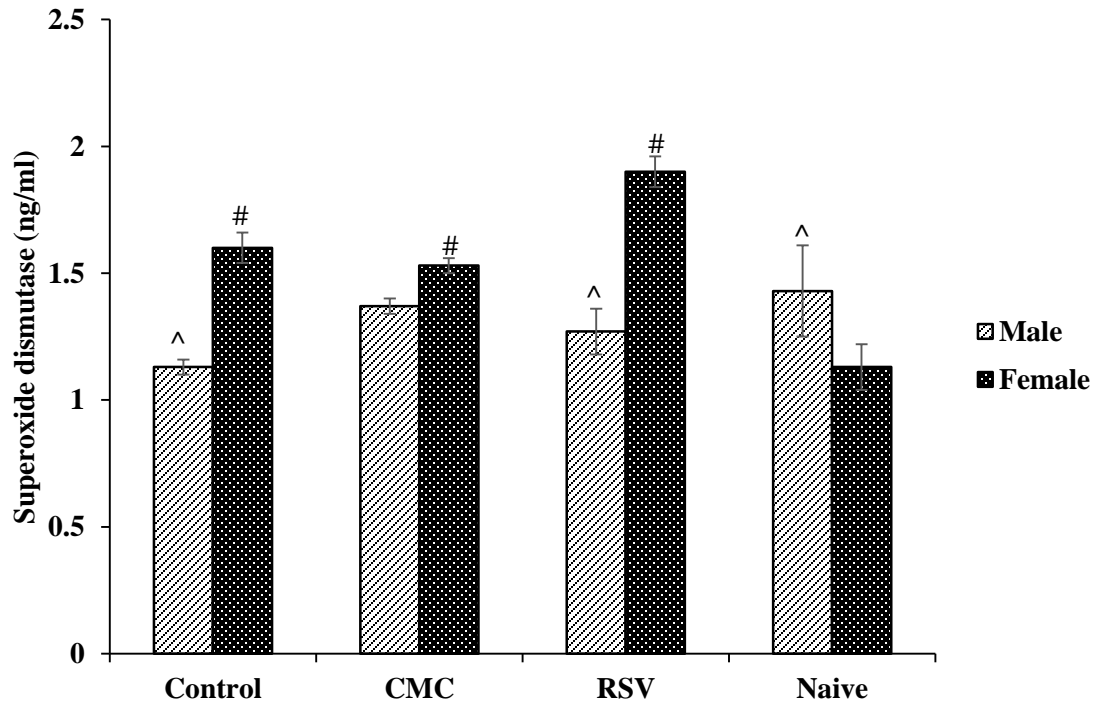


Figure 4.23: Superoxide dismutase concentration in male and female animals subjected to thermal noxious stimulus. # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol.

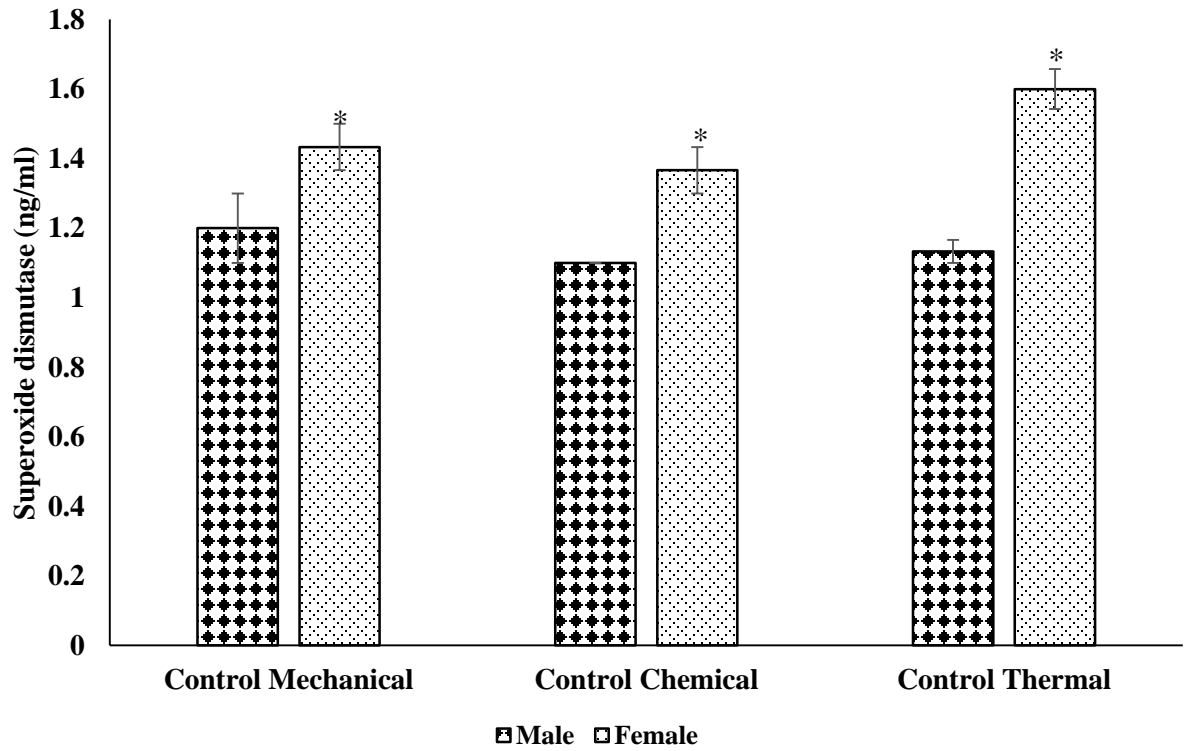


Figure 4.24: Superoxide dismutase concentration in control rats subjected to mechanical, chemical and thermal noxious stimulation during the harmattan season. * male vs. female ($p < 0.05$).

CHAPTER FIVE

5.1 Discussion

This study investigated the influence of the harmattan season, characterized by low ambient temperature in morning and evening hours, but high ambient temperature in the afternoon hours, and low relative humidity, on responses to mechanical, chemical and thermal noxious stimuli in male and female rats and the modulatory effects of resveratrol.

Mechanical, thermal or chemical noxious stimuli often lead to increased synthesis and release of algogenic substances in the periphery, resulting in pain. Mechanical and thermal stimuli are conducted along similar pathways through the thin myelinated A δ nociceptive, while chemical stimuli are mostly conducted through the unmyelinated C fibres. The transduction, processing and perception of these stimuli are different (Meller *et al.*, 1994; Meek *et al.*, 2015). Thus, the study attempted to investigate the influence of environmental factors, sex and resveratrol on these three noxious stimuli.

The results of the experiment generally showed that females had a higher pain sensitivity to mechanical noxious stimulus during the harmattan, it was not statistically significant. The results obtained showed diurnal fluctuations in mechanical pain threshold, decreasing from morning (06:00 h) up to the evening time (18:00 h) in all treatment groups, especially in control animals, and in both sexes. The findings also demonstrated that diurnal fluctuations in response to mechanical noxious stimulation was similar in both male and female rats. The diurnal fluctuations in responses to pain in the present study disagree with those of Strigo *et al.* (2000), who did not observe any influence of ambient temperature on mechanical

sensitivity. The results also agree with the findings of Pechlivanova *et al.* (2010) where diurnal variation obtained in the control animals showed peak thresholds in the morning (09:00 h) and low thresholds in the evening (18:00 h) corresponding to light and dark phase. The differences in the results may be due to the effect of season or ambient temperature, prevailing during the harmattan period of study.

In the chemical assessment of pain using the formalin test, nocifensive behaviour was highest in the CMC group in both the early and late phases of the formalin test, with similar results for both sexes. There are hardly any reports on the effect of CMC in formalin test. The results on CMC obtained in the present study strongly suggest that it may have some synergistic activity with formalin or it modifies pain sensitivity, even though CMC is described as being physiologically inert. Female control animals had higher nocifensive behaviour than the male control animals in both phases of the formalin test.

The formalin test is biphasic, comprising an early (0–5 min) and late (20–30 min) phase (Vogel *et al.*, 2002). The early phase is attributed to C-fibre activation as a result of direct chemical stimulation of nociceptors, while the late phase is dependent on inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Tjolsen *et al.*, 1992; Lee and Jeong, 2002). These functional changes may be initiated by the C-fibre activation during the early phase. The formalin test has been proposed as a model of chronic pain, which is sensitive to centrally-active analgesic agents (Dubuisson and Dennis, 1977).

Ambient temperature has been demonstrated to be a confounding factor in the late phase of formalin test, which may lead to misinterpretations of results and erroneous conclusions (Hole and Tjolsen, 1992). Possible mechanism was that tissue temperature may influence inflammatory process or sensitivity of nociceptors, the involvement of peripheral blood flow may be an additional mechanism (Hole and Tjolsen, 1992). A decrease in temperature have been reported to drastically reduce nocifensive responses in the late phase (Rosland *et al.*, 1991).

Threshold to thermal noxious stimuli, expressed as jumping latency (seconds) in the hot-plate test was similar in both sexes and across treatment groups. Thermal thresholds exhibited a time-dependent increase (0-120 min) at 30 minutes interval in the hot-plate test, but the increase was not statistically significant. Thus, further analysis using linear regression showed a positive linear relationship of thermal threshold with time. This observation was initially thought to result from adaptation, as reported by Hollins *et al.* (2011), that repetitive thermal stimulus of 30 seconds intervals is characterized by adaptation apparently linked to adaptation of A δ nociceptive afferents, followed by sensitisation. In this study, the thermal stimulus was spaced at intervals of 30 minutes to prevent the development of temporal summation, thus excluding adaptation. Interestingly, the results obtained on mechanical pain threshold in this study demonstrated a reverse response for the thermal threshold, with threshold decreasing rather than increasing as was observed in thermal threshold recordings, suggesting the absence of adaptation.

Previous studies have reported time-dependent increase in jump latency in mice (increase in thermal threshold) in the hot-plate test, being highest during the dark phase of the day and lowest at the resting period (Wesche and Frederickson, 1981). Similar findings were also reported by Martinez-Gomez *et al.* (1994) in rats using the tail flick test. Nociceptive flexion reflex induced by an electrical stimulation of the sural nerve was observed to possess circadian variation, with pain intensity being highest in the dark phase (Bourdalle-Badie *et al.*, 1990). Although the present study did not investigate the effect of time of day on thermal sensitivity, the results showed a trend that may be described to increase from the light phase to the dark phase, when the animals were more active. Although, studies in humans have shown that pain threshold to laser induced heat pain do not vary during the day (Bachmann *et al.*, 2011).

Diurnal variation in pain intensity have been previously reported by Glynn and Lloyd. (1975), with the female sex having higher pain ratings than males, apparently due to the subjective nature of pain rating used in the study. Even though, subjectivity plays an integral and important role in pain responses, its exclusion may give an objective assessment of pain because it will exclude social, environmental and cultural influences. Thus, necessitating the use of animals studies (Cobos and Portillo-Salido, 2013).

Results of the present study revealed that resveratrol did not modulate pain threshold when rats were exposed to mechanical, chemical and thermal noxious stimulation. Resveratrol have been reported to inhibit generation of action potentials via modulating the sodium and potassium currents in dorsal root ganglion (DRG) (Granados-Soto *et al.*, 2002; Kim *et al.*,

2005). Furthermore, it has also been reported that intravenous administration of resveratrol suppressed nociceptive sensory transmission in the absence of inflammatory or neuropathic pain (Takehana *et al.*, 2016). These suggest that resveratrol may suppress sensory transmission, including nociception, in both peripheral and central nervous systems. Resveratrol was also demonstrated to attenuate carrageenan-induced hyperalgesia, but did not reverse the swelling and oedema (Gentili *et al.*, 2001). The mechanism was suggested to be related to resveratrol's cyclooxygenase-2 inhibitory property and inhibition of local pain mediators (Gentili *et al.*, 2001; Pharm-Macou *et al.*, 2008; Maia Jr *et al.*, 2012). Resveratrol (50, 100, 200 µg/paw) administered subcutaneously in the right hind-paw also diminished flinching behaviour in the second phase of formalin test but not the first phase (Granados-Soto *et al.*, 2002; Torres-Lopez *et al.*, 2002). Montiel-Ruiz *et al.* (2011) recorded analgesic activity in acetic acid-induced pain following intra-peritoneal administration of 316.2 mg/kg resveratrol. Harb *et al.* (2009) also reported a reduction in acetic acid-induced writhings after oral administration of resveratrol (100 mg/kg).

Our results disagree with the findings reported above, however, it noteworthy that our results agree with the finding of Tsai *et al.* (2012), who showed that resveratrol did not produce anti-nociceptive activity in control animals, although resveratrol was administered intrathecally. Resveratrol administration have also been reported to reverse morphine anti-nociceptive tolerance, however, resveratrol did not affect thermal paw withdrawal threshold in sham operated rats, thus resveratrol is not an analgesic (He *et al.*, 2014).

Inflammation is strongly associated with pain, because the release of inflammatory mediators stimulate and facilitate nociceptors; thus resulting in pain. If inflammation is the source of the pain, then an anti-inflammatory agent will attenuate the pain by removing the source of that pain. The reported activity of resveratrol may have been mistaken for its anti-inflammatory potentials. Another explanation for the reported analgesic activity of resveratrol in the previously reported studies, was that its effects may have been locally exerted evidenced by the route of administration that was used by these studies. Furthermore, resveratrol when administered locally had no analgesic activity in the contralateral limb (Granados-Soto *et al.*, 2002; Torres-Lopez *et al.*, 2002). It suffices to say that the purported analgesic effect of resveratrol is primarily the result of its anti-inflammatory property via modulating the inflammatory cascade by inactivation of cyclooxygenase and down regulation of NF- κ B; thus, resveratrol may serve as a potent anti-inflammatory agent but not an analgesic (Gobec *et al.*, 2015; Tsai *et al.*, 2015).

Sex is an important factor in painful experience (Palmeira *et al.*, 2011). Differences in pain in males and females is strongly associated with subjective component, which could result from emotional experience. Laboratory experiments suggest that women have a lower pain threshold than men, using different noxious stimuli such as heat, cold, pressure and electrical stimulation (Palmeira *et al.*, 2011). Pain is a dynamic phenomenon under the influence of various mechanisms of excitatory and inhibitory control. The differences in pain perception obtained in previously reported studies between sexes may be associated with the hypoactivity of the inhibitory system of pain in females.

Sexual differences in pain have also been attributed to oestrogenic level in humans; low levels are associated with high predisposition to chronic pain; low oestradiol levels promote chronic pain via emotionally-mediated pathway (Rhudy *et al.*, 2013). This finding is further supported by Kowalczyk *et al.* (2010), who reported that menstrual cycles affect pain discrimination, resulting in inconsistencies in pain sensitivities between males and females. The present study did not consider the effect of the estrous cycle or gonadal hormones.

Sodium, potassium, chloride and calcium ions play important roles in pain transduction and signaling (Czeschik *et al.*, 2008; Price *et al.*, 2009; Barragan-Iglesias *et al.*, 2014). Efforts have been focused on these ions and their numerous respective receptor sites as therapeutic targets of pain, and several studies have explored these resources, but the role of electrolytes in pain have not yet been investigated. To the best of our knowledge this is the first account of the investigation on the changes in electrolytes (sodium, potassium, chloride) in animals subjected to mechanical, chemical and thermal noxious stimulations.

In this study sodium ion concentration was significantly higher in the experimental groups when compared to the naïve group. Sodium ion concentrations were also observed to be higher in male naïve animals than female naïve animals. This result agrees with the work of Lasker *et al.* (1985), who reported higher concentration of sodium ion in human males, when compared to females. This result, may be attributed to the difference in activity of Na⁺-K⁺ ATPase in both sexes. A significant inverse relationship has been reported between Na⁺- K⁺ ATPase and erythrocyte sodium concentrations in humans (Lasker *et al.*, 1985). This implies

that sodium homeostasis is not the same in males and females and this observation may contribute to higher predisposition of men to hypertension than women (Lasker *et al.*, 1985).

It is important to mention that there was a significantly higher sodium ion concentration in the control groups that were subjected to mechanical and chemical noxious stimuli than those exposed to thermal noxious stimulus. Similarly, potassium there was a significant increase in potassium in controls, subjected to mechanical than those rats exposed to chemical and thermal noxious stimuli. There was no significant difference in chloride ion concentration in all the groups. The implications of the results are unclear and therefore, require further investigations.

In recent times, oxidative stress has been implicated in the pathogenesis of many disease conditions (Sen *et al.*, 2010; Inanir *et al.*, 2013). When the production of reactive oxygen species (ROS) exceeds the availability of antioxidant systems, a condition defined as oxidative stress is established (Nebbioso *et al.*, 2013). Oxidative stress is an imbalance between increased levels of reactive oxygen species (ROS) and a low activity of antioxidant systems. An increase in oxidative stress induces damage to the cellular structure via lipid peroxidation and potentially destroys tissues. It has been demonstrated that these damaging events are caused by ROS. Endogenous or exogenous antioxidants prevent destructive effect of ROS and RNS (Charles, 2013; Nebbioso *et al.*, 2013).

Painful stimulation increases the production of ROS resulting in lipid peroxidation of cyto-membranes (Rokyta *et al.*, 2003). Studies have established the relationship between pain

and oxidative stress, with MDA concentration reported to increase with increasing pain scores (Slater *et al.*, 2012; Navarro *et al.*, 2013). The ROS sensitizes and activate nociceptive terminals, thus inducing and/or exacerbating pain (Pinho-Ribeiro *et al.*, 2015; Ruiz-Miyazawa *et al.*, 2015). Erythrocytes are highly susceptible to ROS because of their high polyunsaturated fatty acid content, continuous exposure to oxygen and high ferrous ion concentration (Ghorbel *et al.*, 2015; Metherel and Stark, 2016). Thus, they possess cellular defence mechanisms against ROS-induced lipid peroxidation, including enzymatic and non-enzymatic antioxidants (Andriichuk *et al.*, 2016).

The present study demonstrated that resveratrol significantly reduced percentage haemolysis in rats exposed to mechanical stimulation. It also reduced percentage haemolysis in animals subjected to chemical stimulation, however, the reduction was not significant. This implies that resveratrol increased osmotic resistance of erythrocytes to hypotonic solutions, by maintaining membrane integrity of the erythrocytes through the prevention of lipid peroxidation. These findings confirm the potent antioxidant capacity of resveratrol demonstrated by other authors (Gobec *et al.*, 2015; Umeme *et al.*, 2015). Substantial evidences have demonstrated that lipid peroxidation and oxidation of proteins by ROS play a crucial role in oxidative damage in erythrocytes, and may result in modifications in the structural organisation and functions of the cell membrane. The structural modifications may include, increased membrane permeability, decreased membrane fluidity and inactivation of membrane-bound enzymes (Devasena *et al.*, 2001; Filho *et al.*, 2014).

Superoxide dismutase activity concentration in all the rats subjected to the three noxious stimuli did not vary significantly. Interestingly, female control animals had significantly higher superoxide dismutase activity than male control animals, while naïve female animals had lower concentration than male animals, even though MDA levels were similar. This finding is consistent with reports that female animals have a higher antioxidant capacity than male animals (Guevara *et al.*, 2011; Schneider *et al.*, 2014). It has been demonstrated that sex-related differences occur in antioxidant enzyme activity (Geirgiel and Kankofer, 2015). Female animals have been reported to have higher antioxidant enzyme expression and activity, which may be due to the hormonal regulation exerted by estrogens (Vina *et al.*, 2005). Although hormonal influence or oestrus cycle was not investigated in this study, oestrogens have been shown to scavenge ROS directly due to the presence of phenolic ring in their structure as well as via their influence on the regulation of the synthesis and activities of antioxidant enzymes (Pfeilschifter *et al.*, 2002).

Another important finding of the present study was that MDA concentration was significantly higher due to mechanical and chemical noxious stimulation than thermal noxious stimulation. There was a higher percentage haemolysis in the mechanical and chemical noxious stimulation than in the controls subjected to thermal noxious stimulus. Taken together, this result suggests that mechanical and chemical noxious stimuli induce higher oxidative damage than thermal noxious stimulus, though this may be influenced by myriad of factors especially the intensity of noxious stimulation. To the best of our knowledge, this is the first report of increased MDA concentration and erythrocyte haemolysis following mechanical and chemical noxious stimulation.

CHAPTER SIX

6.1 Summary, Conclusion and Recommendations

6.1.1 Summary

In summary, the investigation on nociceptive, oxidative and electrochemical responses of Wistar rats, subjected to mechanical, chemical and thermal noxious stimulations, and the modulatory effects of resveratrol administered orally at 200 mg/kg during the harmattan season in Zaria Nigeria was carried out.

Nociceptive response to mechanical noxious stimulus showed diurnal variation with threshold decreasing throughout the day. There was no significant difference observed in a pain threshold between male and female rats; although, female rats had higher pain responses compared to the male rats. Animals also showed time dependent changes in thermal pain thresholds that were similar in both sexes. Results obtained for chemical noxious stimulation demonstrated increased nocifensive responses in animals administered with the vehicle (CMC), which has not been previously reported. Resveratrol at 200 mg/kg did not significantly modulate pain thresholds in animals subjected to mechanical, chemical and thermal noxious stimuli.

Serum MDA, superoxide dismutase and erythrocyte osmotic fragility were used to assess oxidative status of animals subjected to the various noxious stimuli. The results showed an increase in oxidative disturbance in mechanical and chemical stimulations when compared to thermal noxious stimulation. Superoxide dismutase activities revealed sexual dimorphism, with female animals having higher superoxide activity than male animals.

Sodium ion concentration was significantly higher in animals subjected to mechanical noxious stimulus than naïve animals (animals that were not subjected to any noxious stimulus). Sodium ion concentration was also higher in mechanical and chemical than thermal noxious stimulation. Sodium ion concentration were observed to be higher in male naïve animals than female naïve animals. Potassium ion was significantly higher in animals subjected to mechanical noxious stimulation than animals subjected to chemical and thermal noxious stimulations. No significant change was observed in chloride ion concentration in all the groups.

6.1.2 Conclusions

Mechanical and thermal pain threshold in rats exhibited diurnal variations during the harmattan season. Mechanical, chemical and thermal thresholds in rats is not sex-dependent and were not significantly modified by administration of 200 mg/kg resveratrol. Higher oxidative damage occurred in responses to mechanical and chemical noxious stimulation than thermal noxious stimulation as indicated by the results of erythrocyte osmotic fragility and MDA assessments. Sexual dimorphism was observed in superoxide dismutase activity. Sodium and Potassium ion were also significantly higher in animals subjected to mechanical and chemical than animals subjected to thermal noxious stimuli.

6.1.3 Recommendations

Based on the findings of this study the following recommendations were made:

- i. Similar studies should be carried out during the hot-dry and rainy seasons, to compare and confirm seasonal and diurnal variations in nociceptive response to mechanical, chemical and thermal noxious stimuli.

- ii. Identification of the time when one is most sensitive to pain or when analgesics are most effective may help improve pain management and fixing time of surgeries.
- iii. Electrophysiological studies on major ion channels that contribute to pain should be carried out to assess differences in magnitude of conductance between mechanical, chemical and thermal noxious stimulations.
- iv. Pain receptors such as transient receptor potential receptors should be assessed and their level of activation compared in mechanical, chemical and thermal noxious stimulations.
- v. Further studies need to be done to investigate the relationship between pain and serum electrolytes.
- vi. Detailed investigations on carboxymethyl cellulose need to be done to determine its physiological activities and interactions with other chemicals.

6.1.4 Contributions to knowledge

Based on results obtained in the present study, the following contributions to knowledge were drawn:

- i. To the best of our knowledge this is the first account of this kind that has been reported in literature, where different noxious stimulations were investigated and compared in naïve animals in the tropics.
- ii. Nociceptive responses to mechanical, thermal and chemical pain may be similar in male and female rats and nociceptive responses are similarly affected by hour of the day in males and females [$F(1,24)=1.45, p = 0.47$]; [$F(1,24)= 0.689, p = 0.415$].
- iii. Threshold to mechanical pain [$F(2, 48)=35.8, p = 0.001$] decreases from morning through to the evening.
- iv. Thermal threshold increases in a time-dependent manner [$F(4, 96)=12.47, p = 0.001$] suggesting diurnal characteristics of thermal threshold.
- v. Mechanical and chemical stimulations (47.33 ± 1.45 ; 47.33 ± 1.20) induce higher oxidative damage than thermal noxious stimulations (41.33 ± 0.67).
- vi. Sexual dimorphism exists in serum electrolytes concentrations in rats subjected to different noxious stimulation.

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APPENDICES

Appendix I

Diurnal and sex variations in pain-threshold induced mechanical stimulus in Wistar rats during the harmattan season.

| | | Male | Female |
|---------------------------------|----------------|-------------|-------------|
| 9:00h | Control | 7.81 ± 0.69 | 7.79 ± 0.40 |
| | CMC | 7.09 ± 0.61 | 7.06 ± 0.65 |
| | RSV | 7.26 ± 0.44 | 6.77 ± 0.79 |
| 13:00h | Control | 5.73 ± 0.88 | 5.88 ± 1.49 |
| | CMC | 5.34 ± 0.87 | 4.88 ± 0.62 |
| | RSV | 4.86 ± 0.18 | 4.03 ± 0.32 |
| 18:00h | Control | 4.68 ± 0.45 | 3.96 ± 0.67 |
| | CMC | 4.10 ± 0.45 | 4.67 ± 0.50 |
| | RSV | 4.59 ± 0.36 | 4.15 ± 0.31 |
| Overall (mean ± SEM) | Control | 6.07 ± 0.50 | 5.88 ± 0.67 |
| | CMC | 5.51 ± 0.42 | 5.54 ± 0.50 |
| | RSV | 5.57 ± 0.17 | 4.98 ± 0.31 |

CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix II

Dynamics of time variation in time-course of thresholds of response to thermal pain (hot-plate) stimulus in male and female Wistar rats during the harmattan season.

| | | Control | CMC | RSV |
|-----------------|---------------|-------------|-------------|-------------|
| 0 mins | Male | 1.11 ± 0.12 | 1.22 ± 0.17 | 1.11 ± 0.16 |
| | Female | 1.31 ± 0.17 | 1.31 ± 0.13 | 1.12 ± 0.12 |
| 60 mins | Male | 1.45 ± 0.28 | 1.37 ± 0.14 | 1.40 ± 0.12 |
| | Female | 1.45 ± 0.13 | 1.30 ± 0.08 | 1.19 ± 0.07 |
| 90 mins | Male | 1.64 ± 0.16 | 1.54 ± 0.10 | 1.44 ± 0.16 |
| | Female | 1.40 ± 0.11 | 1.62 ± 0.14 | 1.31 ± 0.07 |
| 120 mins | Male | 1.80 ± 0.19 | 1.67 ± 0.08 | 1.51 ± 0.17 |
| | Female | 1.69 ± 0.18 | 1.84 ± 0.20 | 1.46 ± 0.08 |
| 150 mins | Male | 1.64 ± 0.11 | 1.72 ± 0.16 | 1.90 ± 0.22 |
| | Female | 1.61 ± 0.14 | 1.57 ± 0.21 | 1.38 ± 0.08 |

CMC = Carboxymethyl cellulose, RSV = Resveratrol (200mg/kg). ^{a,b} = means with superscript letters are significantly (P < 0.05) different.

Appendix III

Responses to chemical pain stimulus in male and female rats (n = 5) during the harmattan season (Median).

| | Early phase | | Late phase | |
|----------------|-------------|---------|------------|---------|
| | Males | Females | Males | Females |
| Control | 3.40 | 8.20 | 4.90 | 8.00 |
| CMC | 12.00 | 10.10 | 11.70 | 9.50 |
| RSV | 7.00 | 5.70 | 5.50 | 6.50 |

* indicate significance $P < 0.05$.

Appendix IV

Percentage haemolysis in rats exposed to mechanical noxious stimulation during the harmattan season.

Erythrocyte osmotic fragility (Mechanical) Male

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|---------------|---------------|---------------|--------------|---------------|---------------|
| Control | 0.00 ± 0.00 | 41.67 ± 4.37 | 46.67 ± 3.28 | 56.67 ± 4.18 | 73.33 ± 5.84 | 87.67 ± 2.72 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 48.67 ± 2.40* | 66.67 ± 0.88* | 75.67 ± 0.67* | 83.67 ± 0.67 | 99.00 ± 1.00* | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 41.67 ± 1.76 | 55.33 ± 4.63 | 72.00 ± 3.79* | 86.33 ± 1.33 | 98.67 ± 0.67* | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 29.67 ± 2.91& | 42.00 ± 2.08 | 51.00 ± 1.15 | 67.00 ± 2.08 | 83.00 ± 3.05 | 100.00 ± 0.00 |

Erythrocyte osmotic fragility Mechanical Female

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Control | 0.00 ± 0.00 | 46.00 ± 2.52 | 60.67 ± 4.33 | 77.00 ± 4.58 | 91.67 ± 5.84 | 99.33 ± 0.67 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 47.67 ± 0.88 | 66.33 ± 3.29 | 80.67 ± 4.97 | 92.00 ± 4.16 | 99.00 ± 1.00 | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 37.33 ± 1.20 | 46.00 ± 1.00* | 54.67 ± 0.88* | 69.67 ± 1.45* | 88.00 ± 1.73* | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 30.67 ± 3.53* | 42.33 ± 1.33* | 50.67 ± 1.45* | 69.67 ± 0.33* | 82.67 ± 1.76* | 100.00 ± 0.00 |

* P < 0.05 vs. control; # P < 0.05 vs. naïve. ^ P < 0.05 male vs. female, CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix V

Percentage haemolysis in rats exposed to chemical noxious stimulation during the harmattan season.

Erythrocyte osmotic fragility Chemical Male

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|---------------|--------------|--------------|--------------|--------------|---------------|
| Control | 0.00 ± 0.00 | 41.00 ± 1.16 | 48.33 ± 2.40 | 56.33 ± 3.92 | 72.00 ± 5.86 | 85.67 ± 3.48 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 41.67 ± 1.45 | 50.33 ± 0.66 | 60.67 ± 2.33 | 77.00 ± 4.16 | 92.00 ± 2.00 | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 37.33 ± 2.52 | 42.66 ± 1.20 | 52.33 ± 0.88 | 70.33 ± 0.88 | 84.33 ± 1.45 | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 29.67 ± 5.03* | 42.00 ± 2.08 | 51.00 ± 1.15 | 67.00 ± 2.08 | 83.00 ± 3.06 | 100.00 ± 0.00 |

Erythrocyte osmotic fragility Chemical Female

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|--------------|--------------|--------------|---------------|--------------|---------------|
| Control | 0.00 ± 0.00 | 38.00 ± 3.51 | 48.67 ± 3.76 | 56.00 ± 2.89 | 75.33 ± 0.33 | 89.67 ± 0.67 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 41.00 ± 1.15 | 50.67 ± 2.19 | 58.00 ± 1.53 | 71.67 ± 1.76 | 88.33 ± 3.18 | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 33.00 ± 1.15 | 46.33 ± 0.67 | 55.00 ± 1.52 | 70.33 ± 0.88* | 88.33 ± 1.76 | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 30.67 ± 3.52 | 42.33 ± 1.33 | 50.67 ± 1.45 | 69.67 ± 0.33* | 82.67 ± 1.76 | 100.00 ± 0.00 |

* P < 0.05 vs. control; # P < 0.05 vs. naïve; ^ P < 0.05 male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix VI

Percentage haemolysis in rats exposed to thermal noxious stimulation during the harmattan season.

Erythrocyte osmotic fragility Thermal Male

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|---------------|---------------|--------------|--------------|--------------|---------------|
| Control | 0.00 ± 0.00 | 31.33 ± 0.88 | 41.00 ± 0.58 | 53.33 ± 0.88 | 70.67 ± 1.20 | 88.00 ± 3.00 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 28.00 ± 1.15 | 38.33 ± 1.76 | 51.00 ± 1.15 | 66.67 ± 2.33 | 81.33 ± 0.88 | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 21.00 ± 0.58* | 33.00 ± 1.15* | 49.33 ± 0.88 | 68.00 ± 0.58 | 81.33 ± 0.88 | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 29.67 ± 2.91 | 42.00 ± 2.08 | 51.00 ± 1.15 | 67.00 ± 2.08 | 83.00 ± 5.29 | 100.00 ± 0.00 |

Erythrocyte fragility Thermal Female

| | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|----------------|--------------|---------------|---------------|--------------|--------------|--------------|---------------|
| Control | 0.00 ± 0.00 | 22.00 ± 1.73 | 36.67 ± 2.60 | 50.67 ± 1.45 | 65.00 ± 3.21 | 83.67 ± 0.88 | 100.00 ± 0.00 |
| CMC | 0.00 ± 0.00 | 38.00 ± 1.53* | 48.00 ± 0.58* | 54.67 ± 0.88 | 72.33 ± 3.33 | 91.00 ± 4.58 | 100.00 ± 0.00 |
| RSV | 0.00 ± 0.00 | 30.33 ± 0.88 | 41.67 ± 3.18 | 52.33 ± 1.33 | 68.67 ± 2.02 | 84.67 ± 0.88 | 100.00 ± 0.00 |
| Normal | 0.00 ± 0.00 | 30.67 ± 3.53 | 42.33 ± 1.33 | 50.67 ± 1.45 | 69.67 ± 0.33 | 82.67 ± 1.76 | 100.00 ± 0.00 |

* P < 0.05 vs. control; # P < 0.05 vs. naïve. ^ P < 0.05 male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix VII

Percentage haemolysis in untreated rats exposed to mechanical, chemical and thermal noxious stimuli during the harmattan season.

Control

| | | 0.85% | 0.70% | 0.60% | 0.50% | 0.40% | 0.30% | 0.20% |
|---------------|------------------|-------------|--------------|--------------|--------------|--------------|--------------|---------------|
| Male | Control M | 0.00 ± 0.00 | 41.67 ± 4.37 | 46.67 ± 3.28 | 56.67 ± 4.18 | 73.33 ± 5.84 | 87.67 ± 2.72 | 100.00 ± 0.00 |
| | Control C | 0.00 ± 0.00 | 41.00 ± 1.16 | 48.33 ± 2.40 | 56.33 ± 3.92 | 72.00 ± 5.86 | 85.67 ± 3.48 | 100.00 ± 0.00 |
| | Control T | 0.00 ± 0.00 | 31.33 ± 0.88 | 41.00 ± 0.58 | 53.33 ± 0.88 | 70.67 ± 1.20 | 88.00 ± 3.00 | 100.00 ± 0.00 |
| Female | Control M | 0.00 ± 0.00 | 46.00 ± 2.52 | 60.67 ± 4.33 | 77.00 ± 4.58 | 91.67 ± 5.84 | 99.33 ± 0.67 | 100.00 ± 0.00 |
| | Control C | 0.00 ± 0.00 | 38.00 ± 3.51 | 48.67 ± 3.76 | 56.00 ± 2.89 | 75.33 ± 0.33 | 89.67 ± 0.67 | 100.00 ± 0.00 |
| | Control T | 0.00 ± 0.00 | 22.00 ± 1.73 | 36.67 ± 2.60 | 50.67 ± 1.45 | 65.00 ± 3.21 | 83.67 ± 0.88 | 100.00 ± 0.00 |

Different superscript letters ^{a,b,c} indicate significant difference $p < 0.05$; * male vs. female ($p < 0.05$). M = Mechanical noxious stimulus,

C = Chemical noxious stimulus, T = Thermal noxious stimulus

Appendix VIII

Malondialdehyde concentrations of male and female animals subjected to mechanical noxious stimulation.

| | Male | Female |
|----------------|---------------|---------------------------|
| Control | 47.33 ± 1.45 | 48.33 ± 1.76 |
| CMC | 47.67 ± 1.86 | 46.67 ± 1.45 |
| RSV | 41.00 ± 0.58* | 47.67 ± 2.19 [#] |
| Naïve | 42.00 ± 1.15 | 40.00 ± 0.58* |

* $p < 0.05$ vs. control; [#] $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix IX

Malondialdehyde concentrations of male and female animals subjected to chemical noxious stimulation.

| | Male | Female |
|----------------|---------------|--------------|
| Control | 47.33 ± 1.20# | 42.67 ± 2.19 |
| CMC | 50.67 ± 0.67# | 46.00 ± 1.15 |
| RSV | 47.00 ± 1.54# | 45.67 ± 1.33 |
| Naive | 42.00 ± 1.15* | 40.00 ± 0.58 |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix X

Malondialdehyde concentrations in male and female animals subjected to thermal noxious stimulation.

| | Male | Female |
|----------------|--------------|--------------|
| Control | 41.33 ± 0.67 | 43.33 ± 1.86 |
| CMC | 40.00 ± 2.08 | 42.67 ± 0.88 |
| RSV | 42.00 ± 1.52 | 46.33 ± 3.18 |
| Naive | 42.00 ± 1.15 | 40.00 ± 0.58 |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix XI

Superoxide dismutase activities in male and female rats subjected to mechanical noxious stimulus.

| | Male | Female |
|----------------|-------------|-------------|
| Control | 1.20 ± 0.10 | 1.43 ± 0.07 |
| CMC | 1.33 ± 0.03 | 1.50 ± 0.06 |
| RSV | 1.20 ± 0.06 | 1.57 ± 0.15 |
| Naive | 1.43 ± 0.18 | 1.13 ± 0.06 |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix XII

Superoxide dismutase activities in male and female rats subjected to chemical noxious stimulus.

| | Male | Female |
|----------------|-------------|-------------|
| Control | 1.10 ± 0.00 | 1.37 ± 0.07 |
| CMC | 1.13 ± 0.07 | 1.27 ± 0.09 |
| RSV | 1.07 ± 0.07 | 1.57 ± 0.24 |
| Naive | 1.43 ± 0.18 | 1.13 ± 0.09 |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naïve. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol

Appendix XIII

Superoxide dismutase activities of male and female animals subjected to thermal noxious stimulus.

| | Male | Female |
|----------------|-------------|---------------|
| Control | 1.13 ± 0.03 | 1.60 ± 0.06# |
| CMC | 1.37 ± 0.03 | 1.53 ± 0.03# |
| RSV | 1.27 ± 0.09 | 1.90 ± 0.06*# |
| Naive | 1.43 ± 0.18 | 1.13 ± 0.09*^ |

* $p < 0.05$ vs. control; # $p < 0.05$ vs. naive. ^ $p < 0.05$ male vs. female. CMC = Carboxymethyl cellulose, RSV = Resveratrol