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ACROLEIN-INDUCED EPIGENETIC MODIFICATION OF DNA METHYLATION IN RAT KIDNEY TISSUE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in Graduate School

of Texas Southern University

By

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LIST OF ABBREVIATIONS

5-MC – 5-Methyl Cytosine

5-HmC – 5- Hydroxymethyl Cytosine

8-OHdG -8-Hydroxy-2'-deoxyguanosine

ATSDR - Agency for Toxic Substances and Disease Registry

CFS – Chronic Fatigue Syndrome

Chronic Obstructive Pulmonary Disease (COPD

(cGMP) - Cyclic Guanosine Monophosphate

DNMT - DNA Methyltransferase

TET- Ten-Eleven Translocation Enzymes

HUVEC- Human Umbilical Vein Endothelial Cells

USEPA – United State Environmental Protection Agency

NAC – N-acetyl cysteine

PBS – Phosphate Buffered Saline

IGF - Insulin-like Growth Factor

VSMCs – Vascular Smooth Muscle Cells

TDI - Total Daily Intake

GSH – Glutathione

HPMA - 3-hydroxypropylmercapturic acid

CEMA - 2-carboxyethylmercapturic acid

OPMA - S-(3-oxopropyl)-N-acetylcysteine

WHO - World Health Organization

DNA – Deoxyribonucleic Acid

ROS – Reactive Oxygen Specie

VSMCs- Vascular Smooth Muscle Cells

MESNA - 2-mercaptoethane Sulfonate

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ABSTRACTS:

Mechanism of Acrolein Toxicity: The Effect of Epigenetic Changes. Zivar Yousefipour, Kasturi Ranganna, Mathew Joseph.

MEETING ATTENDED:

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2020 Experimental Biology Orlando Florida (Virtual)

DEDICATION

I dedicate this dissertation to my Lord and Savior Jesus Christ, for his grace, and strength to complete this task and specially to my beloved mum of the blessed memory who raised a strong woman I am today, and I owe all my success to her.

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I give thanks to God Almighty for his mercy that endures forever and gratitude to him for uplifting me academically. To God, be all the glory. To my beloved brother Engr Macmicheal Nwaiwu, I sincerely appreciate your great support (financially and morally), for encouraging me to keep working hard and letting me know that I can do anything I put mind to, thank you so much and God bless you richly. To my younger brothers Darlington and Nelson thank you for your encouragement and prayers. To my dearest uncle and his wife Dr. and Mrs. Osita Opara thank you so much for your support (financially) and your encouragement God bless you.

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ACROLEIN-INDUCED EPIGENETIC MODIFICATION OF DNA METHYLATION IN RAT KIDNEY TISSUE

By

Uchechi Grace Nwaiwu, PhD.

Texas Southern University, 2021

Professor Zivar Yousefipour, Advisor

Acrolein, an alpha-beta unsaturated aldehyde, and a very reactive and toxic compound is released into the environment from different sources. As a pervasive environmental pollutant, acrolein poses a serious environmental health threat acknowledged by investigators, health, and environmental government agencies. Acrolein is released into the environment through the burning of organic compounds. The main sources of acrolein pertinent to human health and toxicity include cooked foods, combustion of fossil fuels, cigarette smoke, overheating of frying oil, endogenously produced via lipid peroxidation, metabolism of polyamine, and anticancer drug cyclophosphamide. Exposure of humans to acrolein is mainly through inhalation of contaminated air, ingestion of contaminated food, and water and dermal contact. Exposure and buildup of acrolein in the biological system have been linked to different disease conditions such as cancer, cardiovascular diseases, multiple sclerosis, spinal cord injury, and Alzheimer's diseases.

Several mechanisms have been proposed for the toxicity of acrolein including inflammation, protein abduction, oxidative stress, DNA abduction, and membrane disruption. Some studies have linked acrolein-induced toxicity to epigenetic modifications. Epigenetics is the modifications in gene expression that do not alter the DNA sequence of a gene. This alteration could occur naturally or by factors such as age, environmental exposure, individual lifestyle, and disease condition. DNA methylation is an epigenetic process that ensues via the addition of a methyl (CH3) group to DNA and frequently alter the role of the gene and transcription factor and as such influence gene manifestation. DNA methyltransferases (DNMTs) are a group of enzymes that control the addition of methyl groups at different levels in the cells. There are three major types of DNMTs; DNMT1 is the methylation enzyme responsible for the maintenance of established patterns of DNA methylation while DNMT3A and DNMT3B mediate the establishment of new

or de novo DNA methylation patterns. On the other hand, Ten Eleven Translocation (TET) enzymes counter the activities of DNA methyltransferases via stepwise biochemical conversion of 5- methylcytosine to 5-carboxyl cytosine, which is then modified by thymine DNA glycosylase (TDG) making it a basic cytosine which allows the regeneration of unmethylated cytosine through base excision repair thereby abolishing DNA methylation.

N- Acetylcysteine (NAC) is an antioxidant that is vital for the reduction of oxidative stress and the downstream negative effects that are associated with it. NAC has shown potency in the inhibition of acrolein-induced toxicity based on the recent studies done in our laboratory and other investigators. NAC beneficial effects are through improvement in the production of cellular glutathione, a natural antioxidant enzyme known to defend the body against the invasion of xenobiotics such as acrolein. Our earlier work using Vascular Smooth Muscle Cells (VSMCs) has indicated that acrolein induces cytotoxicity and modifies histones specifically histone H3, through epigenetic modification via methylation and acetylation and NAC blocks the toxicity and inhibits histone modification. Based on current information, we are hypothesizing that acrolein toxicity is through changes in DNA methylation and NAC prevents this toxicity by inhibiting DNA methylation. Specific aims of the study are: To demonstrate that acrolein induces toxicity in rats is through DNA methylation, Evaluate the activities of DNA methylation/demethylation enzymes, Evaluate the effect of N-acetylcysteine (NAC) on the prevention of DNA methylation and subsequent changes. To investigate our hypothesis, male Sprague-Dawley rats were treated with 2mg/kg/day of acrolein for seven days (i.p) in the presence or absence of 600mg/kg/day of NAC (i.p). The dose and duration of exposure were selected based on our previous studies. At the end of the study period, kidney tissue was collected, nuclear protein and DNA samples were extracted for biochemical analysis. Lipid peroxidation was measured using MDA (Malondialdehyde)-based colorimetric assay. 5-mC hydroxylase TET activity, DNMT activity, Thymine DNA glycosylase activity, DNA Demethylase activity, and DNA Damage Quantification (8-OHdG) were measured by ELISA-based colorimetric and fluorometric assay from Epigentek. Global DNA methylation and global DNA hydroxymethylation were measured by ELISA-based colorimetric assay from Abcam and protein expression by western blotting. Our results revealed that acrolein-treated rats showed about a 169% increase in lipid peroxide level when compared to the control group. The addition of NAC decreases lipid peroxidation level to about 52% compared to the acrolein group. There was about a 67% decrease in 5-mC TET Hydroxylase activity in the acrolein treated group compared to the control group, the addition of NAC increased 5-mC TET Hydroxylase activity by 28% compared to the acrolein group. Consistently, there was about a 14% decrease in demethylase activity in the acrolein group

compared to the control group and the addition of NAC increased demethylase activity to about 20% compared to the acrolein group. Similarly, there was about a 22% decrease in TDG activity in the acrolein treated group compared to the control and the addition of NAC showed an approximately 15% increase in TDG activity compared to the acrolein group. On the contrary, there was about a 30% increase in DNMT activity in the acrolein group compared to the control. The addition of NAC indicated about 32% increase compared to the acrolein group. The reason behind the increase in DNMT activity after the addition of NAC is yet to be understood since NAC is known to repeal acrolein toxicity. There was up to 16% increase in the global 5-mC level in the acrolein treated group compared to the control and the addition of NAC increased it by 16% when compared to the control. The same trend was observed in global 5-HmC where there was a 22% decrease in 5-HmC activity in the acrolein group compared to the control. The addition of NAC increased the level of 5-HmC approximately to 130% compared to the acrolein group. The decrease in global 5-mC and 5-HmC agrees with many investigators' reports (hypomethylation) as the prognosis of different cancer development. Finally, there was about an 18% decrease in the 8-OHdG level in the acrolein group compared to the control and NAC addition increased the 8-OHdG level by 167% compared to the acrolein group. Based on the present data, we are concluding that epigenetic changes observed in acrolein-treated rats are the results of increased generation of ROS, which contribute to increasing oxidation level, alteration of TET enzymes and their products, as well as an increased in DNA methylation and subsequent DNA damage.

CHAPTER 1

INTRODUCTION

1.1 Acrolein

Acrolein, also known as 2-propenal, is an unsaturated aldehyde with a chemical formula of CH_2CHCHO. The compound is a yellow or colorless liquid with unpleasant odor which is reported to be an environmental pollutant due to its widely used in industry.

Acrolein is a prevalent noxious compound which is easily distributed into the environment from several sources. Its ubiquitous presence in our surrounding, presents health hazard to all livings including human and animal. Environmental Protection Agency (EPA) has listed acrolein as a priority toxic compound to the environment (EPA, 2007). According to World Health Organization, the estimated tolerable daily intake (TDI) of acrolein is 7.5mg/kg of body weight (World Health Organization Geneva, 2002).

The Agency for Toxic Substance and Disease Registry (ATSDR) has reported that acrolein minimal acute level centered on respiratory effects on human is 0.007 mg/cm3 or 0.003ppm (ATSDR. 2003).

1.2 Sources of Acrolein

Numerous sources of acrolein is the reason of its widespread in the environment. The exogenous sources of acrolein includes automobile exhaust, forest fires, cigarette smoke, industrial waste. Acrolein is produced in the body through metabolism of polyamines, threonine, and anticancer drug cyclophosphamide (Francis et al., 2013). Acrolein can also

be produced endogenously through the conversion of hydroxy-amino acid (threonine) to acrolein in the present of hydrogen peroxide and chlorine ion. Furthermore, acrolein is produced by copper-dependent amine oxidation of spermidine and spermine via the impulsive retro-Michael addition cleavage (Steven & Maier, 2008). Fried food, alcoholic beverages, charred meat and prolong heating of vegetable oil are the dietary sources of acrolein.

1.3 Metabolism of Acrolein

Assimilation of acrolein into the body system is due to its high solubility nature. Acrolein combines with important cellular antioxidants examples as thioredoxin and glutathione and forms conjugates. Glutathione S-transferase catalyzes conjugation of glutathione by acrolein. The resultant product of acrolein combination with glutathione can be broken down by different enzyme reaction to form different harmful products. For instance, acrolein conjugate with glutathione forms 2-carboxyethyl mercapturic acid (CEMA) via aldehyde dehydrogenase breakdown, forming a known urinary byproduct S-(3-hydroxypropyl)- mercapturic acid (3-HPMA) by Aldo-keto reductase (Mohammad et al., 2015). In addition, metabolites of acrolein can further undergo oxidation and epoxidation reaction with conjugates of glutathione to yield a carcinogenic compound, glyceraldehyde, recognized to be deleterious to human health (Smita et al., 2015). The byproduct of acrolein interaction with biological nucleophiles has been linked to harmful effect of acrolein to human and animals (Stevens & Maier, 2015).

1.4 Uses of Acrolein.

The high industrial demand for acrolein has increased the production of acrolein to over 500, 000 tons per year (Nauman et al., 2014; Journal of Catalysis 2016). The different uses of acrolein include as a micro biocide in an oil well, as a slimicide in paper manufacturing, and as a warning agent in methyl chloride refrigerant. Acrolein is used in the production of poisonous gas mixtures for military purposes, as a coolant in water towers, in the manufacture of plastics and perfumes, production of pesticides , acrylic acid, as an algaecide and herbicide in irrigation canals, used to produce colloidal forms of metals, and as a liquid hydrocarbon fuel.

1.5 Exposure and Health Impacts of Acrolein

U.S Environmental protection Agency has cataloged acrolein toxicity as a main concern to environmental health. In united states, acrolein has been known to present a health risk, and discovered to be in a substantial amount in many schools and homes (Logue et al., 2012). Exposure of human to acrolein could be via airborne, occupational, and dietary exposure. The common routes of exposure include inhalation, ingestion and contact with the skin. Prolonged cigarette and hookah smokers, as well as people living in densely polluted environment and firefighter are more predisposed to acrolein exposure (Daffa et al., 2014). Contact of acrolein with different organs of the body such as the skin, eye, lung, kidney, and liver, has been reported to cause various harmful effects such as redness, irritation, inflammation, congestion etc. Also, elevated acrolein adducts have been reported to be the underlying pathology of numerous ailments linked to oxidative

stress such as Sjogren's syndrome, autoimmune disorder, cerebral stroke, and infarction (R. Saiki et al., 2011). Uncleared acrolein conjugate with glutathione could result in covalent protein adducts, causing compromised protein structure and function. Buildup of such adduct and or misfolded proteins can induce ER stress, and acrolein-induced ER stress is shown in vitro in various cell types (Jingwen et al., 2016).

High level of 3-HPMA (acrolein byproduct) in urine has been seen in stroke patient and decreased level of 3-HPMA has been reported to reduce the development of stroke (Mikamia et al., 2012). Research has shown that reduced level of glutathione in the nervous system is due to the formation of GSH-acrolein conjugate which results in the aggravation of stroke (Takahiro et al., 2012). In addition, several chronic disease conditions such as various types of cancer, Alzheimer's, and cardiovascular disease, neurological, nephrological and hepatological toxicity, spinal cord injury, multiple sclerosis, and diabetes mellitus has been connected to acrolein by multiple reports from investigators (Akshata et al., 2015).

A study by Katia et al., (2014) reported detection of the exact damaging agents in cigarette smoke (CS) responsible for reduced Salivary lactate dehydrogenase (LDH) enzyme activity and mechanisms involved in CS oxidative damage. Purified LDH samples was subjected to separate levels of CS, acrolein. acetaldehyde, peroxynitrite and RNS donors. Response of these various exposures was assessed by spectrophotometric enzyme activity assay and western blot analysis. The result showed that CS exposure resulted in 34% in LDH activity, exposure to 10 µmol of acrolein caused 61% reduction, while acetaldehyde, peroxynitrite and RNS donors exposure resulted in no significant

effect. The carbonyl immunoblotting assay showed a fourfold increase in carbonyl content when treated with CS and a sevenfold increase when treated with acrolein, which revealed that acrolein, an alpha-beta-unsaturated aldehyde, was the major component of CS responsible for reduced salivary LDH activity.

Previous work in our laboratory, using rat models treated with 2 and 4 mg acrolein/kg body weights, reported elevated blood pressure due to a reduction in nitric oxide availability, a rise in lipid peroxidation, and a decrease in Cyclic guanosine monophosphate (cGMP). The authors concluded that the increase in blood pressure was a result of changes in the level of GST and GST-Px detoxifying enzymes that inflated vascular response to phenylephrine (Yousefipour et al., 2005).

In another study, Wei Yang Chen et al., (2016) examined the pathogenic role of acrolein in hepatic endoplasmic reticulum (ER) stress, steatosis, and injury in experimental Alcohol Liver Disease (ALD). In vitro hepatoma H4IIEC cells and in vivo chronic plus binge alcohol feeding in C57Bl/6 mice models were used to examine alcohol-induced acrolein accumulation and consequent hepatic ER stress, apoptosis, and injury were examined both in vitro and in vivo. The result showed alcohol intake and metabolism resulted in hepatic accumulation of acrolein-protein adducts, by up-regulation of cytochrome P4502E1 and alcohol dehydrogenase, and down-regulation of glutathione-s-transferase-P, known to breakdown or detoxifies acrolein. In addition, alcohol-induced acrolein adduct accumulation led to hepatic ER stress, proapoptotic signaling, steatosis, apoptosis, and liver injury. Damage to DNA as well as DNA repair proteins leading to abnormal repair and increases in cells and mutational vulnerability has been reported to

have connection with acrolein interaction. Although, these effects might amount to acrolein lung carcinogenicity, the machineries by which acrolein induces lung diseases excluding cancer are undefined. (Hsiang-Tsui Wang et al., 2017).

A previous study done in our laboratory using Vascular smooth muscle cells has shown that acrolein causes cytotoxicity through epigenetic modification of H3 histones via epigenetic modification mechanism (methylation and acetylation) and as such alters gene expression (unpublished data).

CHAPTER 2

LITERARY REVIEW

2.1 Acrolein Mechanism of Toxicity

A strong electrophile, acrolein possess high affinity to bind and react with endogenous nucleophiles such as proteins, DNA, glutathione etc. The interaction of acrolein with cellular molecules are the major pathways for acrolein toxicity. This can occur due to reaction with proteins resulting in protein abduction and binding to DNA leading to abnormal DNA abduction. Studies have reported different mechanisms that acrolein exert toxicity. Such mechanisms includes oxidative stress, inflammation, endoplasmic reticulum stress and mitochondrial stress (Akshata et al., 2015). Acrolein causes damage to the body by weakening the body immune system via the inhibition of immune system (suppression of NF-kB protein complex) from countering the invasion of virus and bacteria attack as well as harmful pollutants.

2.2 Acrolein Causes Inflammation

Inflammation is a process of body immune response against harmful attacks such as injuries, infection, and toxins with the purpose to repair or heal. Prolonged inflammation occurs when the immune response persist exposing the body in a steady state of awareness. The healing response requires the release of important endogenous molecules such as proteins, antibodies, and elevated blood flow. It has been echoed in many investigations that one of the common acrolein mechanism of toxicity is the induction of

inflammation through enhancing the generation of ROS and acting as a free radical. Acrolein has been reported to provoke pulmonary inflammation as well as lung epithelial cells death through the generation of NF-κB signaling in a mouse *in vivo* study (Sun Y et al., 2014). Acrolein also triggers NF-kB pathway and stimulate pro-inflammatory cytokines including cyclooxygenase-2 (Sarkar et al., 2007). Furthermore, acrolein induces *TNF-α, IL-6,* and *IL-8* mRNA expression via NF-κB stimulation in human umbilical vein endothelial cells (HUVECs) (Haberzett et al., 2004). A research was done to expose acrolein proinflammatory ability in primary nasal epithelial cell (PNECs) along with nicotine and acetyl aldehyde. PNECs from 19 healthy subjects were grown in submerged cultures and incubated with acrolein, nicotine, and CSE preceding to stimulation with Pseudomonas aeruginosa lipopolysaccharide (PA LPS). The data showed that four-hour exposure of 30 μM of acrolein to PNECs resulted in two-fold increase in IL-8 (pro-inflammatory) and increase release of IL-8 after stimulation with PA LPS compared to nicotine anti-inflammatory 0.6-fold release of IL-8 after 50μM exposure to nicotine for 24 h, which exposes acrolein pro-inflammatory ability in PNEC cultures (David M. et al., 2014).

2.3 Protein Abduction by Acrolein

High Reactivity of acrolein with important biological molecule such as protein leads to abnormal protein function. For instance, acrolein reacts with protein residues such as cysteine, lysine, and histidine of the peptide chain through Michael-type addition. The resultant lysine abduct, N-(3-formyl-3, 4-dehydropiperidino) FDP-Lys is an unstable intermediate that covalently attached to thiols for example, glutathione via

reserved electrophilic carbonyl moiety (Aldini et al., 2011). The interaction of FDP-Lys, glutathione and acrolein is found to diminish cellular glutathione, an important antioxidant principally known to combat acrolein assault, thus, increasing the level of oxidative stress and as such impinge body defense mechanism (Luo & Shi, 2005).

Acrolein has be found to play an vital part in the emergence of systemic maladies, examples are neurodegenerative diseases (Wood et al., 2007), cardiovascular diseases (DeJarnett et al., 2014), and diabetes (Daimon et al., 2003).

A study reported that acrolein FD-Lys abduct was found in high concentration in the vitreous fluid of patients with Proliferative Diabetic Retinopathy (PDR). High level FD-Lys was also seen in glial and endothelial cells of the fibrovascular tissues obtained from patients with PDR indicating acrolein play a part in the pathogenesis of diabetes retinopathy (Miyuki et al., 2020).

2.4 DNA Abduction by Acrolein

Reactive oxygen species generation by acrolein via reaction with DNA which could lead to gene mutation as well as carcinogenicity. Another way acrolein induce toxicity is through reaction with deoxy guanosine (dG) producing two cyclic DNA adducts, a- and c-hydroxy-1, N2-propano-20-deoxyguanosine leading to mutagenicity at a different level in cell. Formation of DNA adducts with acrolein have been described *in vitro* and have also been identified *in vivo* in several different animal, human tissues, and cells (Franco et al., 2011). Acrolein is also known to provoke DNA damage as well as inhibit the repair by impeding repair enzymes possibly by acrolein-adduction resulting in an enhanced

proteasome-dependent deprivation (Wang *et al.*, 2012). CpG sites are known to be susceptible to acrolein-DNA adduction, elevated acrolein-induced G-to-T and G-to-A mutation occurrence to levels found in the CpG sites in the p53 gene, is recognized to be mutational hot spot in tobacco smoke-associated lung cancer (Wang *et al.*, 2013).

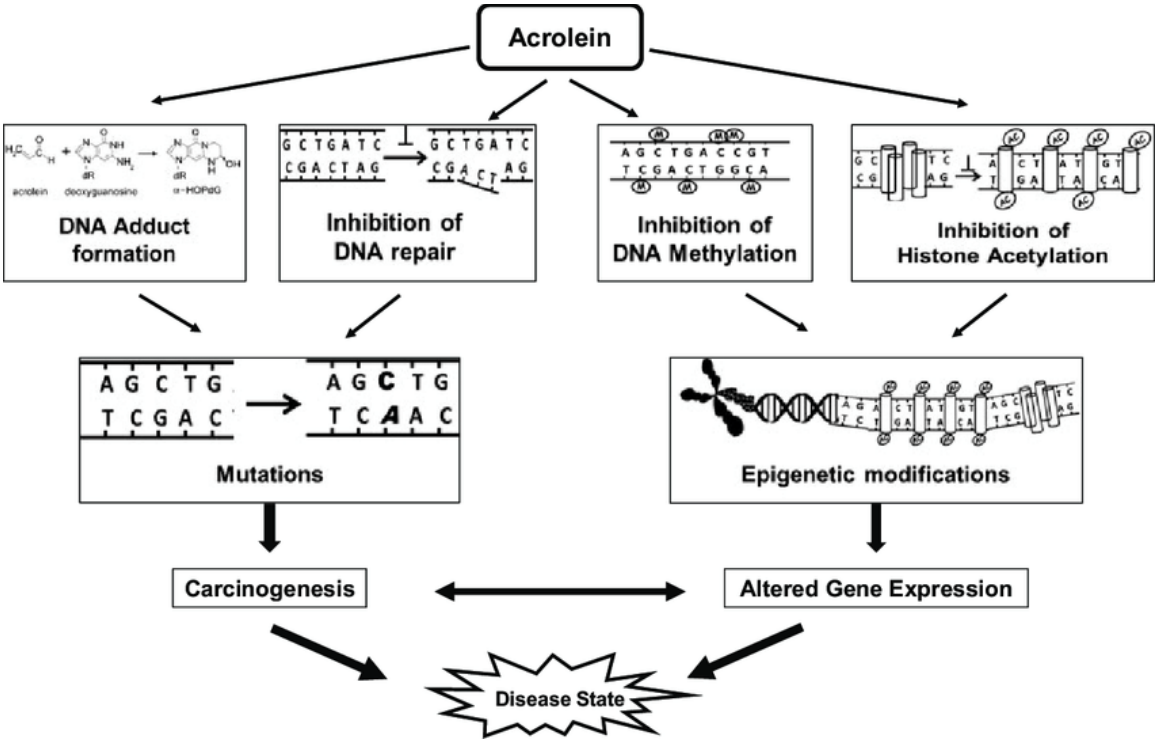


***Figure 2.1*: *Schematic Representation of Acrolein Effects on DNA* (S.Joshi-Barve, 2016).**

2.5 Oxidative Stress

Oxidative stress is the generation of free radicals and the inability of body antioxidants to inhibit the harmful effects of these reactive oxygen species (ROS). ROS is known to be

reason behind different physiological functions such as cell signaling and are usually produced as a byproduct of oxygen metabolism as well as environmental pollutants.

Acrolein reaction with cellular lipid leads to production of free radicals which contributes to increased oxidative stress. It has been reported that acrolein reduction of endogenous glutathione results in the elevated oxidative stress and cell death in retinal microvascular endothelial cells and glial cells (Murata et al., 2017 & 2019). In addition, spermine oxidation by VAP-1 or SMOX leads to acrolein generation and aggravation of oxidative stress in the micro-environment either by restricting the antioxidant defense system or increasing generation of reactive oxygen species (Wu D et al., 2020).

An investigation was carried out by (Masanori et al., 2019) to examine the pathological effects acrolein of in COPD development. Acrolein concentration was assessed in plasma from 47 patients with COPD and 18 current smokers without COPD, and in supernatants of homogenized lung tissues obtained from 10 never-smokers, 8 current smokers, and 8 patients with COPD by high-performance liquid chromatography. The level of oxidant and antioxidant activity were quantified using derivatives of reactive oxygen metabolite (d-ROM) and bio-antioxidant power (BAP). The result indicated that plasma acrolein were significantly high in COPD patients' smokers than non-COPD smokers confirming heightened acrolein concentrations were highly implicated in the pathogenesis of COPD through meddling in the balance of oxidative stress as against antioxidant potency.

2.6 Epigenetics

Epigenetics is the change in phenotypic features of an individual without changing the genotypic constituents of the individual that is liable for how cells read the genes. There are different ways through which epigenetic adjustments can ensue. It either arise naturally or can be determined by different reasons such as age, environmental exposure, individuals' lifestyle, and disease state. Epigenetic alteration happens in such a way that the cell differentiates in a manner that results in liver cells, brain cells, and skin cells. Dysfunctional epigenetic modification can result in disease conditions, an example is cancer.

2.6.1 DNA Methylation

DNA Methylation is the most popular mechanism of epigenetic modification where a methyl group (CH_3) is attached to the segments of DNA. The attachment results in the turning off the genes, silencing or inactivating it thereby inhibiting the production of protein from the modified gene. It could also lead to abnormal gene transcription which is the chief cause of genetic disorders such as cancer, cardiovascular and respiratory diseases, metabolic and degenerative disorders. (Santos et al., 2007).

Methods of DNA methylation at cytosine residues, precisely at CpG sites, is a lively and revocable epigenetic modification (Ramchandani et al., 1990). In human genome, clusters of CpG sites regions are known as CpG Island that are normally found in closeness to or within transcription start site (TSS) along with the promoters of housekeeping and/or tissue-specific genes (Gardiner et al., 1987). DNA Methyl transferase enzymes also

known as DNMTs are group of enzymes that control the addition of methyl groups in different levels in cells. There are three major types are of DNMTs, DNMT1 are the methylation enzymes responsible for the maintenance of established patterns of DNA methylation while DNMT3A and DNMT3B mediate establishment of new or *de novo* DNA methylation patterns. Addition of a methyl group to cytosine at its fifth carbon, creating 5-methylcytosine (5-mc), by the three major DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) are implicated to be the basis of repression of linked genes as this mark and about of transcription start site (TSS), has been associated to transcriptionally repressed chromatin that rejects access for transcription factors, such as serum response factor (SRF) (Jones, 2012).

DNA Methylation through DNMT1 has shown to play essential role in the development of gastrointestinal smooth muscle cells of rats and diseases which is demonstrated in study done by Brian G. Jorgensen et al., (2018), the study was done using DNMT1-knockout mice and disease human GI tissue. The outcome validates that DNA hypomethylation in embryonic smooth muscle cells (ESMC), via congenital DNMT1 deficiency, contributes to massive dysregulation of gene expression and is lethal to GI-SMC.

The outcomes also propose that DNMT1 has an essential role in the embryonic, primary development process of SMC with consistent patterns being found in human GI diseased tissue. Chromatin remodeling, histone modifications, and non-coding RNA are other forms of epigenetic mechanism.

Understanding genetic processes and how environmental factors affect the phenotypic features of a gene became a concern which bring about clarification of Conrad Waddington (1942) molecular basis of epigenetics. His observation unraveled how environmental stress impacted on the physical features in Drosophila fruit flies. From then on, research endeavor has paid attention to scrutinizing epigenetic mechanism in relation to different epigenetic changes that have occurred and how it relates to human disorders and disease conditions.

In 1969, Griffith and Mahler proposed that DNA methylation can be essential in long-term memory function (Holliday, 2006). A distinctive epigenetic point was identified because of mild DNA methylation of insulin-like growth factor II (IGF2) gene (Heijmans et al., 2008). Similarly, famine prenatally exposed adults were also recognized to have a notable occurrence of schizophrenia (St Clair et al., 2005).

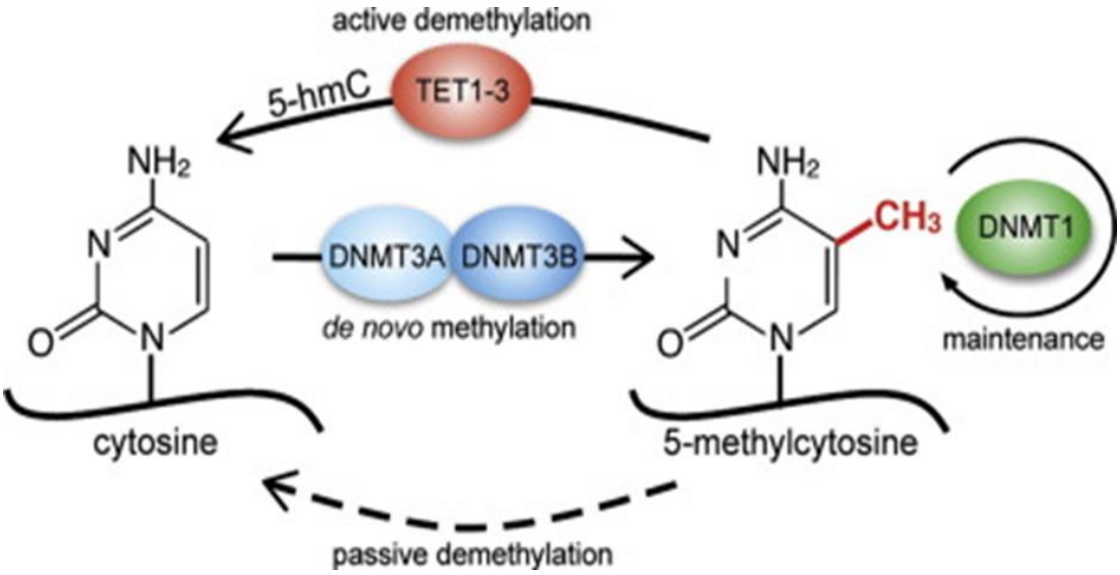


Figure 2.2: Schematic Representation of DNA Methylation **(Christina Ambrosi et al., 2017).**

Phenotypic changes that occur in individual adult life is because of prenatal and postnatal environmental exposures interfering with epigenetic marks which control the information that is stored in the genome. A study conducted by Waterland et al, (2003) reported that administering a methyl donor supplement (vitamin B12, betaine choline, and folic acid) to a woman during gestation period can affect the phenotype of the offspring (viable yellow agouti) through direct alteration of the epigenome (Dolinoy et al., 2006). Studies conducted using animal models has shown that short-term environmental exposure results in prolonged alteration of epigenetic marks that have lasting phenotypic aftermath (Sinclair et al., 2007; Waterland et al., 2003). Many researchers investigated embryonic development because it is the essential stage of initiating and maintaining epigenetic marks (Reik et al., 2001). In fact, culturing preimplantation of mice zygote shows that

epigenetic marks are prone to a nutritional condition in the early period of life development (Morgan et al., 2008; Doherty et al., 2000).

2.7 Ten Eleven Translocation (TET) Enzymes

TET enzymes are part of dioxygenases family known to be chiefly responsible for oxidizing methylated cytosine. Research has shown important roles of TET enzymes in various biological and pathological processes that ranges from regulation of DNA demethylation, gene transcription, embryonic development, and oncogenesis (Xiaotong Yin et., 2016). The three major member of this family are ten-eleven translocation methyl cytosine dioxygenase 1 (TET1), methyl cytosine dioxygenase (TET2), and methyl cytosine dioxygenase (TET3). TET 1 gene was initially acknowledged when it was determined to be glued to the Mixed Lineage Leukemia (MLL) gene due to a translocation event that happened between chromosomes ten and eleven henceforth the name (Lorsbach RB et al., 2003).

The key role of these three forms of TET enzymes is their involvement in biochemical pathway that converts 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) and subsequently regulates the conversion of 5hmC to 5-formylcytosine (5fC) and then 5fC to 5-carboxylcytosine (5caC). Even though experimental data shows that TET3 does so to a lesser extent than TET1 and TET2. (He YF et al., 2011). Despite the fact that TET enzymes were formerly believed to be foundation of DNA damage. Despite the fact that TET enzymes were formerly believed to be foundation of DNA damage, Novel studies have implied that this catalytic activity could essentially be the initial steps of DNA

demethylation process. The demethylation process begins with the changing of 5-mC to 5caC after numerous rounds of oxidation by TET enzymes, followed by the elimination of the modified cytosine base by thymine DNA glycosylase (TDG) which leaves an abasic site on the DNA. Finally, base excision repair occurs where a new unmodified cytosine is restored at the site, thus completing the process of DNA demethylation (Kohli RM et al., 2013). Disturbance of epigenetic patterns as well as DNA methylation forms, leads to the bedrock of cancer development. DNMT3A gene is an example of somatic mutations in gene that codes for the machinery that bring about DNA methylation have been causally connected to malignant transformation.

One of the remarkable facts about TET enzymes is their involvement in the elimination of epigenetic mark, which has appeared to be a vital mechanism in tumor cancer suppression. It appears that the three TET genes are altered and demonstrate lessened expression, and the proteins has decreased activity in a wide range of dissimilar cancer types. Therefore, specific regulation of DNA methylation forms, which is somewhat mediated by TET enzymes, is significant for typical growth and provides an ultimate defense against cellular transformation (Kasper and Kristian, 2016).

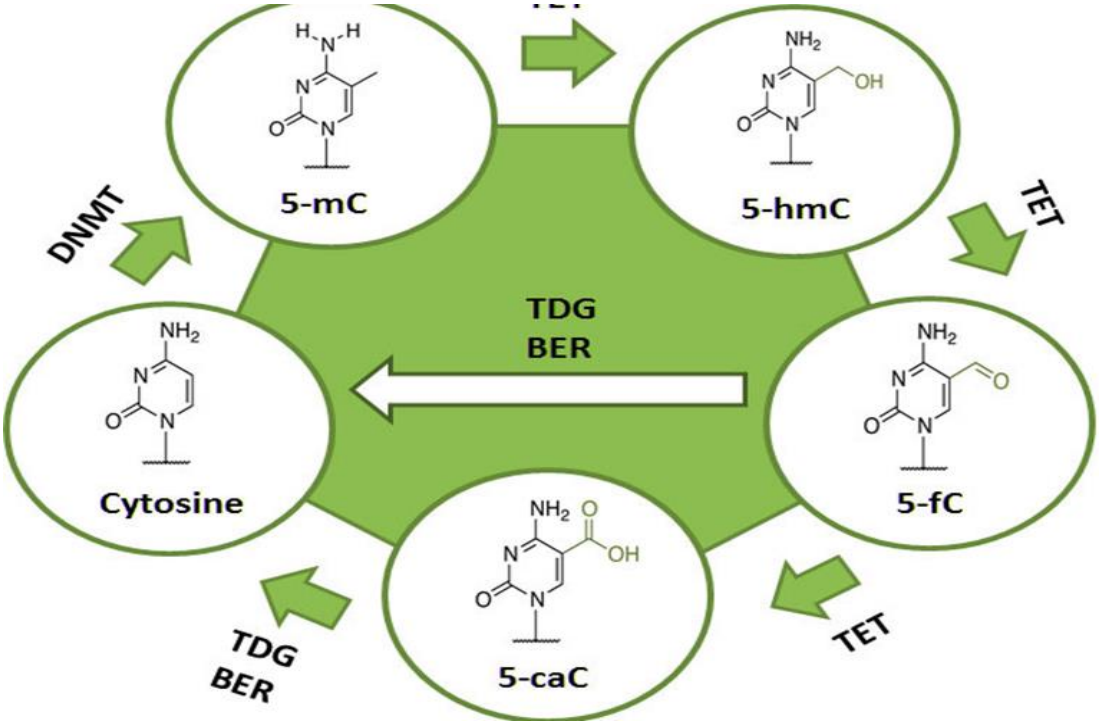


Figure 2.3: Description of Cytosine's Methylation and Demethylation processes. Adjusted Forms of Cytosine Along with Resultant Enzymes Responsible for each Modification by **(What is epigenetics.com)**

2.8 DNA Demethylase

Demethylase enzymes has been discovered to catalyzes hydrolytic removal methyl residue from methylated DNA. Research has also shown that DNA demethylase completely removes methylated and hemi-methylated DNA revealing dinucleotide specificity as well as elimination of mCpG sites in diverse sequence framework. It has been reported that demethylase is a processive enzyme even though its limiting factor in methyl residue elimination is the initiation of demethylation and is ascertained through sequence characteristics. (Nadia et al., 1999).

A recent study Xirong Lu et al, 2020 explored the inhibition of NF-kB by oleanolic (OA) acid to downregulate PD-L1 by promoting DNA demethylation in gastric cancer cells focusing on epigenetic regulation using MKN-45 cells. MKN-45 cells were treated with IL-1β (20 ng/mL) and/or OA at 30 and 40 μM for 24 hours. Measurements of DNA demethylase activity was measured using 10 μg of nuclear protein extracts with the EpiQuik DNA Demethylase Activity/Inhibition Assay kit. The findings demonstrated that DNA demethylase activity showed high DNA demethylation states upon IL-1β exposure, which was reversed by OA, implying an involvement of DNA methylation/demethylation confirming that decreases DNA demethylation contributed to OA down regulation of PD-L1 in IL-1β-treated gastric cancer.

2.9 N-Acetylcysteine

N- acetylcysteine (NAC) is an effective free radical scavenger known to stimulate the production of endogenous glutathione. NAC is an optimal sulfhydryl group whose redox

state has demonstrated to be advantageous to human health by reason of its antioxidant function to cells against damaging foreign bodies and other toxic pollutants. N-acetylcysteine is a derivative of amino acid residue L-cysteine. NAC is an antioxidant that has been used extensively in therapy for several illnesses such as acetaminophen poisoning, kidney and lung diseases, heart attack, stroke, epilepsy seizures, bipolar disorder (Magalhaes et al., 2011 ; Briguori et al., 2002 ; Perry & Shannon, 1998 ; Bibi et al., 1992). Studies has shown the usefulness of NAC in the abrogation of oxidative stress induced by acrolein. NAC has shown to alleviate the biomarkers of oxidative stress in cholestatic animal (Ali et al., 2019). In addition, NAC has been reported to prevent acrolein-induced cytotoxicity via the enhancement of endogenous glatathione level (Milton et al., 2015).

A research was carried out to investigate the effect of acrolein and chloro-acetaldehyde (CAA) on human urothelial cells in vitro and to evaluate the effectiveness of NAC against acrolein and CAA toxicity, the study used human urothelial cells (RT4 and T24) the cells were treated with acrolein or CAA. The alterations in cell viability, reactive oxygen species, caspase-3 were quantified. The result revealed that NAC significantly protected the human urothelial cells against decrease cell viability, increased ROS production induced by acrolein and CAA. (Kylie et al., 2019).

Another investigation was done to assess the role of NAC treatment on biomarkers of oxidative stress and organ histopathological changes in a rat model of cholestasis/cirrhosis. Bile duct ligation (BDL) animals were exposed to100 and 300 mg/kg of NAC through I.P for 42 days. oxidative stress biomarker in the liver, brain,

heart, skeletal muscle, lung, serum, and kidney tissue, and organ histopathological variations were examined. The data showed a substantial increase in reactive oxygen species, lipid peroxidation, and protein carbonylation were identified in various tissues of BDL rats. The study also revealed that tissue antioxidant capacity was impeded, glutathione (GSH) reservoirs were reduced, and oxidized glutathione (GSSG) levels were considerably enhanced in the BDL group. Substantial tissue histopathological changes were apparent in cirrhotic animals. Addition of NAC substantially mitigated biomarkers of oxidative stress and abrogate tissue histopathological alterations in cirrhotic rats. These results demonstrated that NAC is a possible protective treatment for cirrhosis and the subsequent obstructions (Mohammad et al., 2021).

Arwa Alnahdi et al., (2019) carried out an experiment to evaluate the molecular mechanisms of oxidative stress, and inflammatory and antioxidant responses in the presence of high concentrations of glucose and fatty acids aa well as quantify the effect of N-acetyl cysteine protective ability on β-cell toxicity. An insulin-secreting pancreatic β-cell line (Rin-5F) were used in the study. The data illustrated that the cellular and molecular mechanisms underlying β-cell toxicity are caused by elevated oxidative stress, imbalance of redox homeostasis, glutathione metabolism and changes in inflammatory responses. Addition of NAC decreased oxidative stress and modifications in GSH metabolism linked with β-cells cytotoxicity.

A 2020 review done by Zhongcheng, and Carlos stated that NAC could combat COVID-19. NAC is identified for its anti-inflammatory and immune-modulating characteristics and a powerful precursor for cellular glutathione which is known to protect the body

system against harmful effect based on the pathway of toxicity. NF-kB activation is the pathway for the replication of human coronaviruses (HCoV-229E). Since NAC is recognized to suppress activation of NF-kB, this implies that administration of NAC could reduce the replication rate of COVID 19 and as such reduced disease severity. In addition, a cohort study done by (Ibrahim et al., 2020) showed that intravenous administration (IV) of NAC considerably ameliorate disease conditions in 10 severe respirator-dependent COVID-19 patients, aged from 38 to 71 years, including one with Glucose-6-phosphate dehydrogenase (G6PD) deficiency. IV administration of NAC decreased inflammatory markers, for example C-reactive protein (CRP) and ferritin, and similarly enhanced lung functions. Eight out of ten patients were released, and the other two patients displayed better condition before the study was published. Therefore, concluded that this clinical practice additionally verifies the efficacy of NAC in COVID-19 treatment.

CHAPTER 3

MATERIALS AND METHOD

3.1 Ethical Aspect of Research

This study was approved by Texas Southern University Institutional Animal Care and Use Committee (IACUC) and adhered to National Institutes of Health (NIH) guideline and animal welfare act for handling laboratory animals. Male Sprague Dawley were used for this study. There were no use of human or germ cells.

3.2 Materials

Male Sprague Dawley rats were purchased from ENVIGO (Indianapolis, United States), Acrolein from Sigma-Aldrich Chemicals (St Louis, MO), BCA protein assay kit from Pierce (Rockford, IL, USA), EpiQuik Nuclear Extraction Kit, EpiQuik DNMT Activity/Inhibition Assay Ultra Kit, Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit, Epigenase Thymine DNA Glycosylase (TDG) Activity/Inhibition Assay Kit, EpiQuik DNA Demethylase Activity/Inhibition Assay Ultra Kit, EpiQuik 8-OHdG DNA Damage Quantification Direct Kit all from EPIGENTEK (Epigentek Group Inc. 110 B County Blvd. Ste 122 Farmingdale, NY 11735 United States). 1 x Global DNA Methylation Assay Kit and Global DNA Hydroxymethylation Assay Kit from ABCAM. Odyssey classic from Licor for immunoblotting, Tissue-Lyser (Qiagen), Centrifuge (Eppendorf), and holding and metabolic cages.

3.3.1 Treatment

Male Sprague Dawley (300-400 g, ENVIGO) were treated with or without acrolein (2 mg/kg/day,7 days) and NAC (600 mg/kg/day, 10 days). The control group was subjected to the same experimental protocol and received equal amount of vehicle. Animals were weighed at the start of treatment, labeled, and put in holding cages. Urine samples were also collected before and after treatment. Animals had free access to food and water during the duration of treatment according to approved animal care protocols.

A total of 28 rats were divided into four groups. Group one was the control (wild Type) which was treated for 7 days with 400 ul of phosphate buffer saline (PBS) and 3.7% of DMSO via intraperitoneal injection (I.P). Second group was treated with acrolein (2 mg/kg/day for 7 days, via I.P). The third group was treated with 600 mg/kg/day of N-acetyl cysteine (NAC) via I.P for 10 days. The fourth group was treated with acrolein (2 mg/kg, 7 days, I.P) plus 600mg/kg/day of NAC for 10 days via I.P (400 µl) (Table 3.1). Addition of acrolein treatment started after three days of treatment with NAC. All treatment dilutions followed approved recommendation from manufacturer.

Experimental Design

Table 3.1

Group NO.	Group Name	Treatment Concentration	Route of Exposure	Number of Rats	Exposure Duration
1	Control	400µg PBS, 3.7% DMSO	I.P	7	7 Days
2	Acrolein	2mg/kg/day	I.P	7	7 Days
3	NAC	600mg/kg/day	I.P	7	10 Days
4	ACR + NAC	2mg/kg +600mg/kg NAC	I.P	7	Ist 3 days NAC only then 7 NAC +ACR

3.3.2 Collection of Organs and Body Fluid

Before and at the end of the treatment period, animals were placed in metabolic cages for 24 hours for urine sample collection. Blood was collected in anesthetized rats and rats were sacrificed at the end. Liver, heart, kidney, and aorta were harvested, rinsed, and immediately frozen at -80 °C according to approved protocols. kidney tissue was used for biochemical analysis.

3.4 Lipid Peroxidation

Levels of lipid peroxidation was measured using ABCAM Lipid Peroxidation (MDA) Assay Kit (Cambridge, MA) following manufacturers according to supplier's protocol. The kit directly measures malondialdehyde (MDA) the biomarker of lipid peroxidation. 50µl of kidney homogenate was used for the assay. The reagents were added to 96-well plates. Plates were incubated for 60 minutes, and absorbance was read at 695nm per supplier protocol.

3.5 Global DNA Methylation

Global DNA methylation was measured using abcam global DNA methylation Assay kit (5 methyl cytosine) (Cambridge, MA) according to the manufacturer's protocol. DNA was isolated from Sprague-Dawley rats tissue using Qiagen AllPrep DNA/RNA/Protein Mini Kit (Germantown, MD). DNA concentration was measured using Nanodrop One Technology from Thermo Fisher Scientific (Carlsbad, CA). 100ng of purified DNA were used to perform the ELISA assay. Absorbance was read at 450 nm on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). Global DNA methylation quantification was calculated as ng/percentage. Data are expressed in percentage difference over unexposed (control) DNA lysates.

3.6 Global DNA Hydroxymethylation

Global DNA Hydroxymethylation was measured using abcam global DNA Hydroxymethylation Assay kit (5hmc) (Cambridge, MA) according to the manufacturer's protocol. DNA was isolated from Sprague-Dawley rats tissue using Qiagen AllPrep

DNA/RNA/Protein Mini Kit (Germantown, MD). DNA concentration was measured using Nanodrop One Technology from Thermo Fisher Scientific (Carlsbad, CA). 100ng of purified DNA were used to perform the ELISA assay. Absorbance was read at 450 nm on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). Global DNA Hydroxymethylation quantification was calculated as ng/percentage. Data are expressed in percentage difference over unexposed (control) DNA lysates.

3.7 DNA Damage Quantification

DNA damage quantification was measured using EpiQuik 8-OHdG (8-hydroxy-2'-deoxyguanosine) DNA Damage Quantification Direct Assay (Farmingdale, NY) according to the manufacturer's protocol. DNA was isolated from Sprague-Dawley rats tissue using Qiagen AllPrep DNA/RNA/Protein Mini Kit (Germantown, MD). DNA concentration was measured using Nanodrop One Technology from Thermo Fisher Scientific (Carlsbad, CA). 100ng of purified DNA were used to perform the ELISA assay. Absorbance was read at 450 and 655 nm on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). DNA damage quantification was calculated as ng/percentage. Data are expressed in percentage difference over unexposed (control) DNA lysates.

3.8 DNMT Activity

DNMT activity was measured using the Epigentek EpiQuik DNMT Activity/inhibition Assay Ultra kit (Colorimetric and Fluorometric method) (Farmingdale, NY) according to the manufacturer's protocol. Kidney tissue were used to extract nuclear protein, Nuclear

lysates were prepared using Epigentek Nuclear Extraction kit (Farmingdale, NY) and protein concentration was measured using the BCA assay from Thermo Fisher Scientific (Carlsbad, CA). 10µg nuclear proteins were used to perform the ELISA assay. Absorbance was read at 450 and 655 nm for Colorimetry and 530_{ex}/ 590_{em} nm for Fluorometry on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). DNMT activity was calculated as ng/min/mg. Data are expressed in percentage difference over unexposed (control) nuclear lysates.

3.9 5-mC Hydroxylase TET Activity

Total TET activity was measured using the Epigentek Epigenase 5mC Hydroxylase kit (Colorimetric and Fluorometric; Farmingdale, NY) according to the supplier's protocol. Kidney tissue were used to extract nuclear protein, Nuclear lysates were prepared using Epigentek Nuclear Extraction kit (Farmingdale, NY) and protein concentration was measured using the BCA assay from Thermo Fisher Scientific (Carlsbad, CA). 10µg nuclear proteins were used to perform the ELISA assay. Absorbance was read at 450 and 655 nm for Colorimetry and 530_{ex}/ 590_{em} nm for Fluorometry on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). Hydroxymethylated product formed was measured within a standard curve (0–2 ng), TET activity was calculated as ng/min/mg. Data are expressed in percentage difference over unexposed (control) nuclear lysates.

3.10 DNA Demethylase Activity

DNA Demethylase activity was measured using the Epigentek EpiQuik DNA Demethylase Activity/inhibition Assay Ultra kit (Farmingdale, NY) according to the manufacturer's protocol. Extract nuclear protein, Nuclear lysates were prepared using Epigentek Nuclear Extraction kit (Farmingdale, NY) and protein concentration was measured using the BCA assay from Thermo Fisher Scientific (Carlsbad, CA).10µg nuclear proteins were used to perform the ELISA assay. Absorbance was read at 450 and 655 nm on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). DNA Demethylase activity was calculated as ng/min/mg. Data are expressed in percentage difference over unexposed (control) nuclear lysates.

3.11 Thymine DNA Glycosylase Activity (TDG)

TDG activity was measured using Epigentek Epigenase TDG activity/inhibition assay kit. (Farmingdale, NY) according to the manufacturer's protocol. Kidney tissue were used to extract nuclear protein, Nuclear lysates were prepared using Epigentek Nuclear Extraction kit (Farmingdale, NY) and protein concentration was measured using the BCA assay from Thermo Fisher Scientific (Carlsbad, CA).10µg nuclear proteins were used to perform the ELISA assay. Absorbance was read at 450 and 655 nm on a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). TDG activity was calculated as ng/min/mg. Data are expressed percentage difference over unexposed (control) nuclear lysates.

3.12 Statistical Analysis

Data are expressed as mean± SD from two or three independent experiments as indicated in figure legends. t test and one-way analysis of variance (ANOVA) were used for comparison of groups and between treatments for significant difference. Statistical significance was ascertained by using Graph Pad Prism version 9.10 software from Graph Pad Software, Inc. (La Jolla, CA, USA). P value $\leq$ 0.05 were considered significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Changes in Animal Weight

In this study, 28 male Sprague Dawley rats were divided into four groups: control group, NAC group (600mg/kg/day, 10 days), acrolein treated group (2mg/kg/day, 7 days), and acrolein plus NAC group for the seven-day treatment. At the end of the treatment, there were no significant difference in animals' weight before and after treatment (Table 4.1).

4.2 Lipid Peroxidation

Malondialdehyde (MDA) is a biomarker for lipid peroxidation. To demonstrate that acrolein induce toxicity in rats and show if NAC could reverse the toxicity, levels of lipid peroxidation products were measured in rat kidney homogenate. Measurement of Lipid peroxides indicated the group treated with acrolein had an approximately 169% increase in MDA level when compared with the untreated control. The addition of NAC showed an approximately 52% decrease in lipid peroxidation (MDA) level when compared with the acrolein group (Figure 4.1).

4.3 Global DNA Methylation (5-mC) Assay

To measure the level of global 5-mC in acrolein-induced DNA methylation and disclose whether NAC could hinder the effect of acrolein and thus, reverse DNA methylation. 5-mC level was measured using Global DNA Methylation (5-mC) Assay kit. 100ng of DNA sample from rat kidney tissue was used following the manufacturer's protocol. The

measurement of 5-mC level was done within the groups after treatment. The group treated with acrolein indicated up to 15% increase in 5-mC level when compared with the untreated control. The addition of NAC resulted in approximately 16% increase in 5-mC level compared to acrolein group (Figure 4.2).

4.4 Global DNA Hydroxymethylation (5-HmC)

To measure the level of global 5-HmC in acrolein-induced DNA methylation and reveal whether NAC could hinder the effect of acrolein and thus, reverse DNA methylation. 5-HmC level was measured using Global DNA Hydroxymethylation (5-HmC) Assay kit. 100ng of DNA sample from rat kidney tissue was used following the manufacturer's protocol. The measurement of 5-HmC level was done within the groups after treatment. The result revealed that group treated with acrolein indicated approximately up 22% decrease in 5-HmC level compared to the untreated control. The addition of NAC showed approximately 130% increase in 5-HmC level compared to the acrolein group (figure 4.3).

4.5 DNA Damage Quantification

To quantify DNA damage in acrolein treated rats and whether NAC could thwart the effect of acrolein we measured DNA damage. DNA damage quantification was measured using EpiQuik 8-OHdG (8-hydroxy-2'-deoxyguanosine) DNA Damage Quantification Direct Assay kit. 100 ng of DNA extracted from rat kidney tissue was used following the manufacturer's protocol. The measurement of DNA damage was done between the groups at the end of the treatment. Results indicated approximately up to 18% decrease in

8-OHdG level in rats treated with acrolein compared to the untreated control and to our surprise addition of NAC further increased that by 167% when compared with the acrolein group (Figure 4.4).

4.6 DNMT Activity

In this assay, we explored the role of DNMT enzyme in acrolein induced DNA methylation and whether NAC could hinder the effect of acrolein. DNMT Activity was measured using EpiQuik DNMT Activity/Inhibition Ultra Assay kit. 10µg nuclear extract from rat kidney tissue was used following the manufacturer's protocol. The measurement of DNMT activity was done between the groups after treatment. The group treated with acrolein resulted in 30% increase in DNMT activity when compared with the untreated control and addition of NAC showed approximately 32% increase DNMT activity compared to the acrolein group (Figure 4.5).

4.7 5-mC Hydroxylase TET Activity

In this study, we hypothesized that exposure to acrolein will affect DNA methylation and interaction of acrolein with DNA methylation will contribute to acrolein-induced toxicity.

Therefore, in our study, we evaluated the role of TET enzymes in DNA methylation/demethylation in differently treated groups of rats. 5mC Hydroxylase TET Activity was measured using Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay kit. 10 µg nuclear extract from rat kidney tissue was used for the analysis following the manufacturer's protocol. Comparison of 5mC Hydroxylase TET activity was done between the groups after treatment. We found out that tissue DNA methylation

had a negative association with acrolein exposure. In other hands, the group treated with acrolein indicated up to 33% decrease in 5mC Hydroxylase TET activity when compared with the untreated control. Furthermore, we saw addition of NAC resulted in approximately 28% increase in 5mC Hydroxylase TET activity compared with acrolein group (Figure 4.6).

4.8 DNA Demethylase Activity

To evaluate the effect of acrolein and NAC on DNA demethylase, Demethylase activity was measured using EpiQuik DNA Demethylase Activity/Inhibition Assay Ultra kit. 10 µg nuclear extract from rat kidney tissue was used following the manufacturer's protocol. The measurement of demethylase activity was done within the groups after treatment. The results indicated an approximately 14% decrease in demethylase activity in the acrolein-treated group compared to the untreated control. The addition of NAC showed approximately 20% increase demethylase activity compared to the acrolein group (figure 4.7).

4.9 Thymine DNA Glycosylase Activity (TDG)

In this experiment, we wanted to assess the role of TDG enzyme in acrolein induced DNA methylation/demethylation and to find out whether N-acetyl cysteine could avert acrolein effect and impede DNA methylation. TDG activity were measured using Epigenase Thymine DNA Glycosylase Activity/Inhibition Assay kit. 10µg nuclear extract from rat kidney tissue was used following the manufacturer's protocol. The measurement of TDG activity was done within the groups after treatment. The result

showed that group treated with acrolein indicated approximately 22% decrease in TDG activity when compared with untreated control, the addition of NAC showed an approximately 15% increase in TDG activity compared to the acrolein group (Figure 4.8).

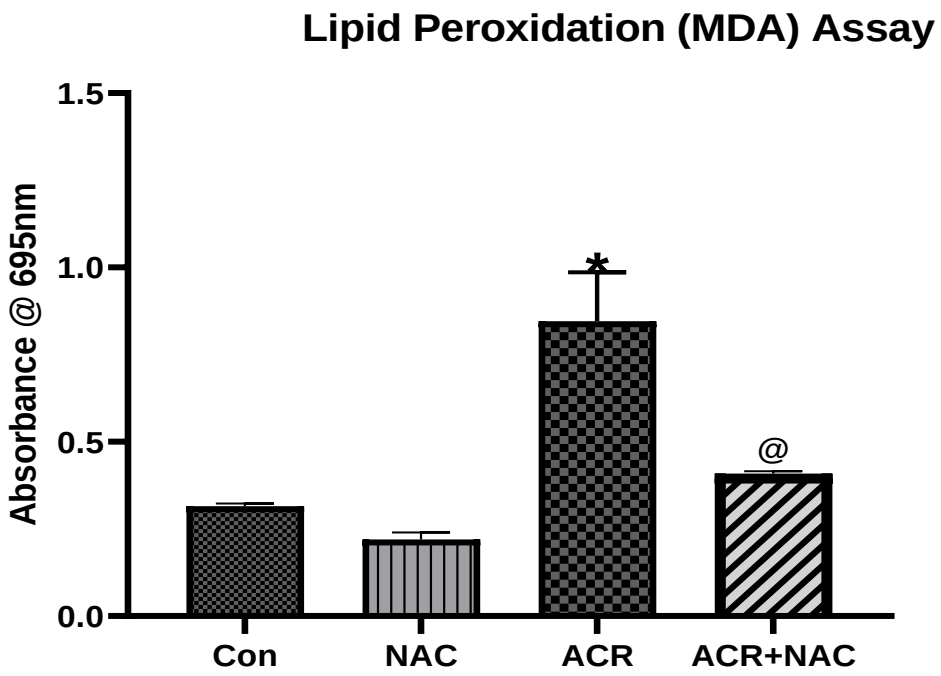
Changes in animal weight

Table 4.1 Mean animal body weight in grams (g) After 7 days Exposure

Group	Treatment/ Concentration	Weight before Treatment	Weight after Treatment	Percentage Difference
1	CON (400µg 3.7% DMSO)	335.8±20.9	348±22.12	4%
2	NAC (600mg/kg/day)	283±8.45	296±9.40	5%
3	ACR (2mg/kg/day)	331±21.95	344±22.23	4%
4	ACR+NAC (2mg/kg+600mg/kg NAC)	388±8.28	399±8.50	3%

Aim 1: To show acrolein induced toxicity in rat kidney tissue.

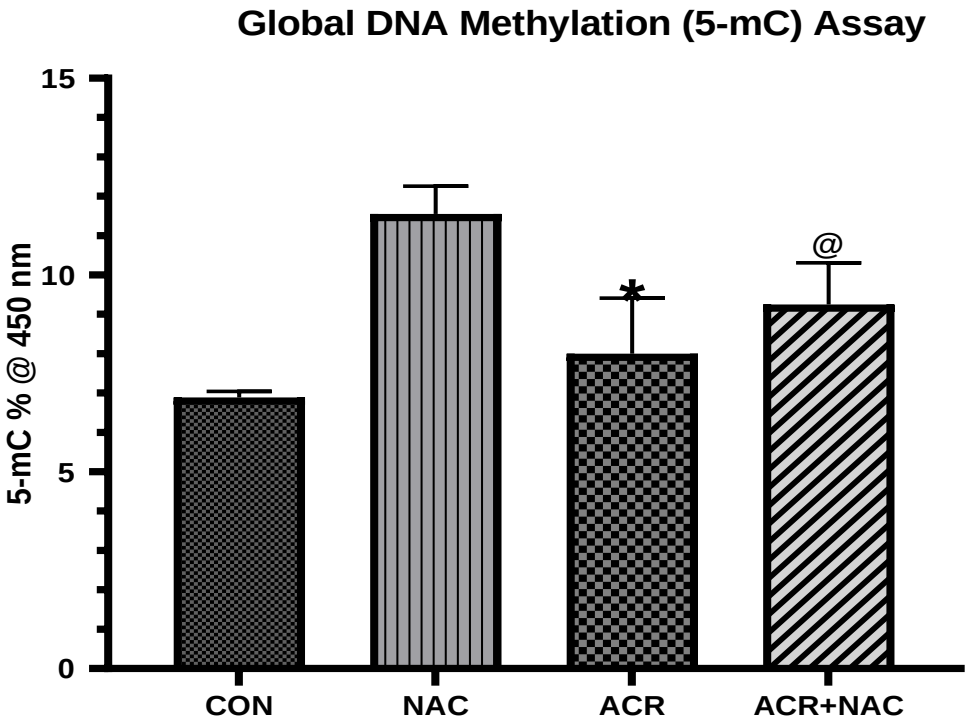
Figure 4.1 Effects of Acrolein/ NAC Treatment on Rat Kidney Tissue



Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. 50µl of Kidney tissue homogenate was used to measure lipid peroxidation. Levels of lipid peroxides from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean $\pm$SEM. (error bars) *$P \leq 0.05$ compared to Con, $^{@}P \leq 0.05$ compared to ACR. Levels of lipid peroxidation activity showed significant (*$P \leq 0.0215$ and $^{@}P \leq 0.082$).

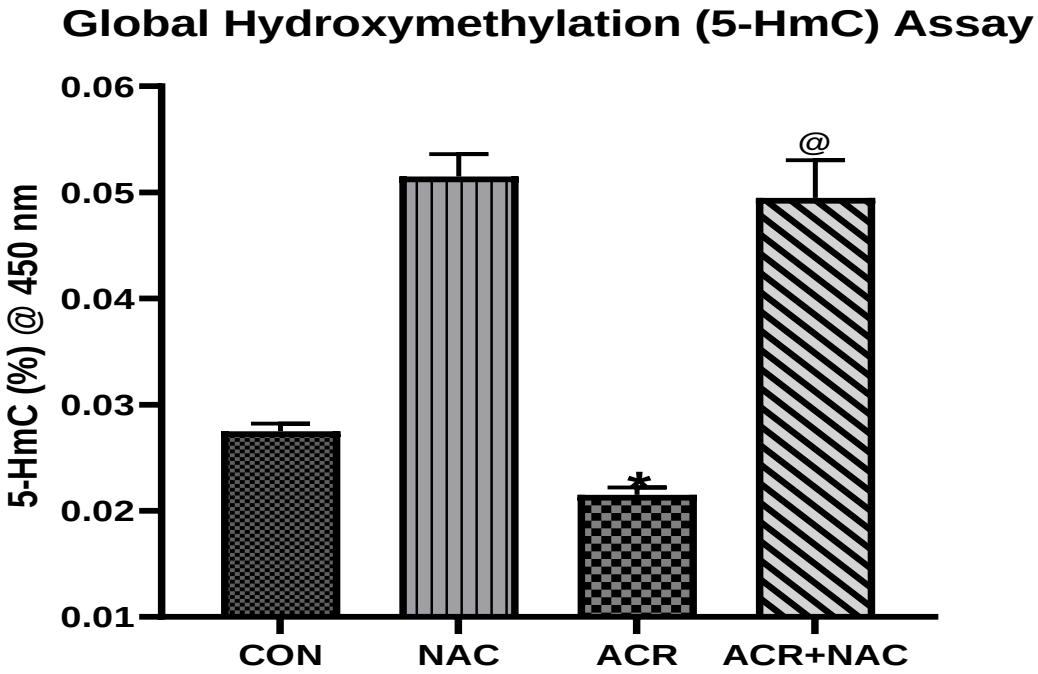
Aim 2: Demonstrate that acrolein induce toxicity in rats is through DNA methylation and the resulting DNA damage, Assays carried out include:

Figure 4.2: Effects of Acrolein/ NAC Treatment on Global DNA Methylation 5-mC



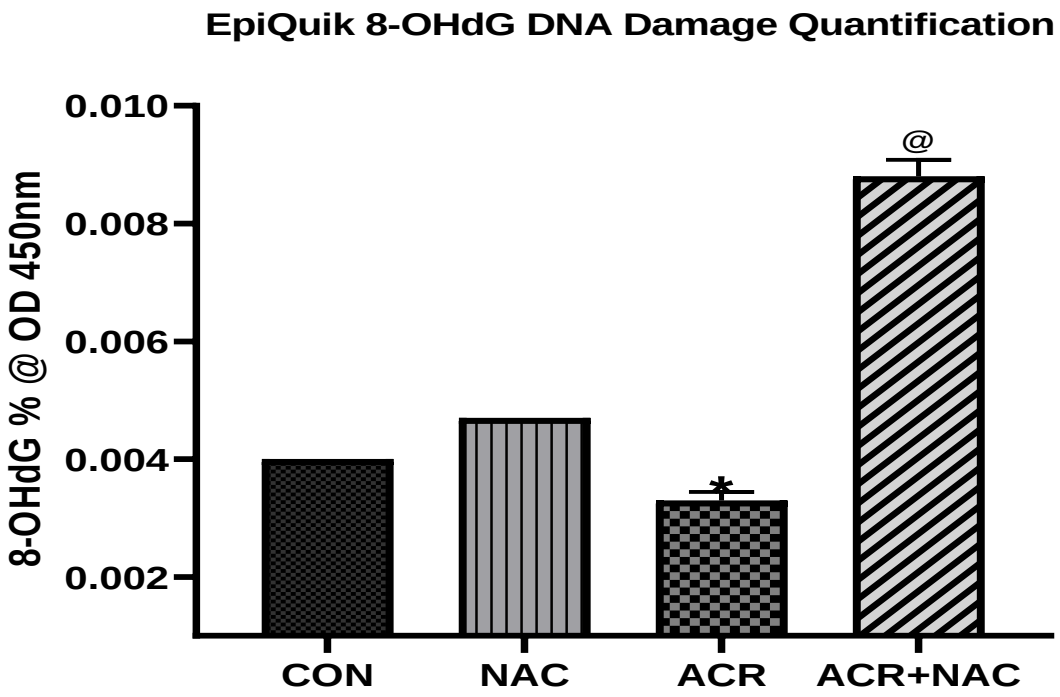
Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. 100ng of DNA sample from rat kidney tissue was used to measure 5-mC level. Level from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean $\pm$SEM. (error bars*)* *$P \leq 0.05$ compared to Con, $^{@}P \leq 0.05$ compared to ACR. 5-mC level showed significant (*$P < 0.0001$ and ns $^{@}P \leq 0.0917$).

Figure 4.3: Effects of Acrolein/ NAC Treatment on Global Hydroxymethylation (5-HmC)



Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. 100ng of DNA sample from rat kidney tissue was used to measure 5-HmC level. Level from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean ±SEM. (error bars) *P ≤0.05 compared to Con, $^{@}$P≤0.05 compared to ACR. 5-HmC level showed significant (*P ≤ 0.0215 and $^{@}$P ≤ 0.0082).

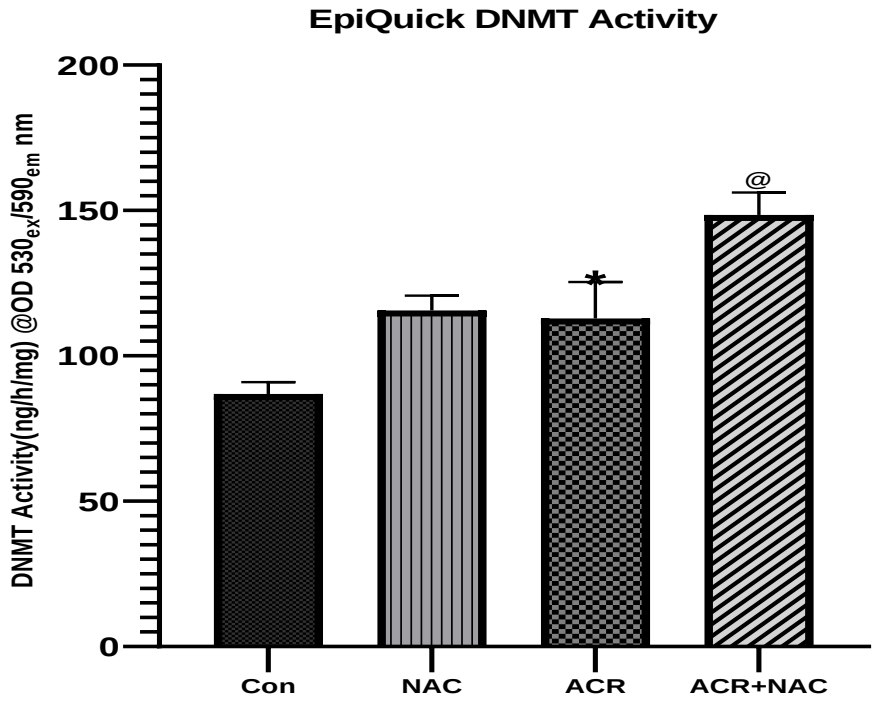
Figure 4.4: Effects of Acrolein/ NAC Treatment on level of 8-OHdG



Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. 100ng of DNA sample from rat kidney tissue was used to measure 8-OHdG level. Level from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean $\pm$SEM. (error bars*)* *$P \leq 0.05$ compared to Con, $^{@}P \leq 0.05$ compared to ACR. 8-OHdG level showed significant (*$P \leq 0.0198$ and $^{@}P \leq 0.0016$).

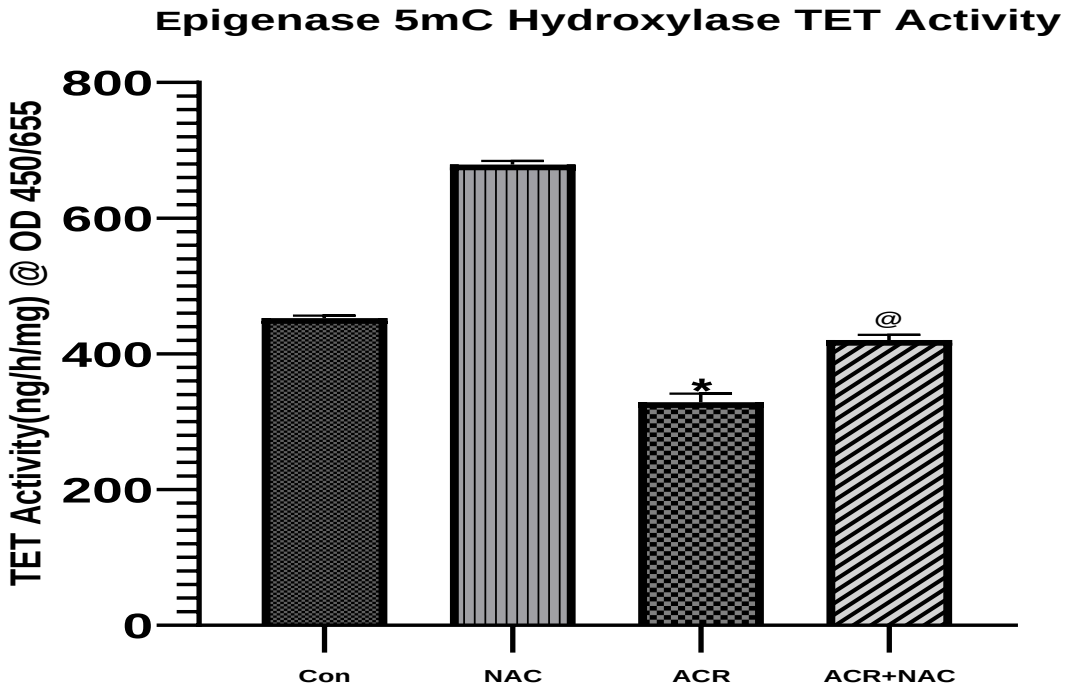
Aim 3: Evaluate the activities of DNA methylation/demethylation enzymes. Assays carried out include:

Figure 4.5: Effects of Acrolein/ NAC Treatment on DNMT Activity



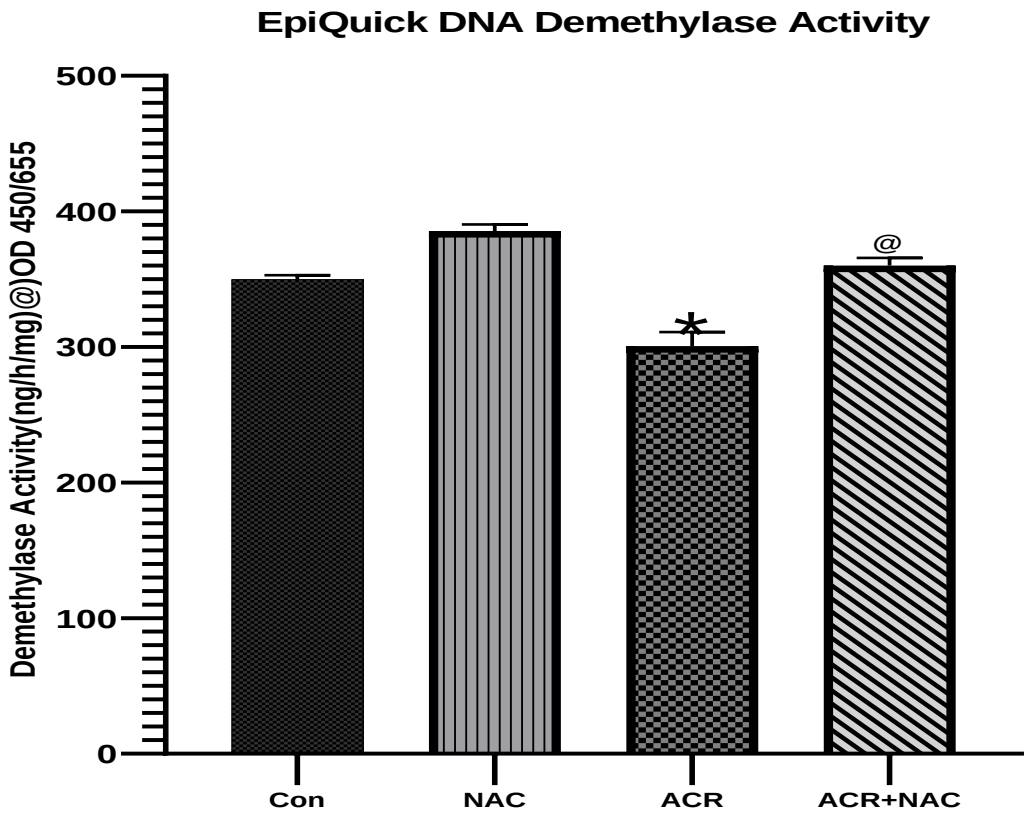
Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. Kidney tissue was used to extract nuclear protein (10µg) for DNMT activity. Activity from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean ±SEM. (error bars*)* *P≤ 0.05 compared to Con, $^{@}$P ≤ 0.05 compared to ACR. DNMT activity showed significant (*P ≤ 0.0074 and $^{@}$P ≤ 0.0099).

Figure 4.6: Effects of Acrolein/ NAC Treatment on TET Activity



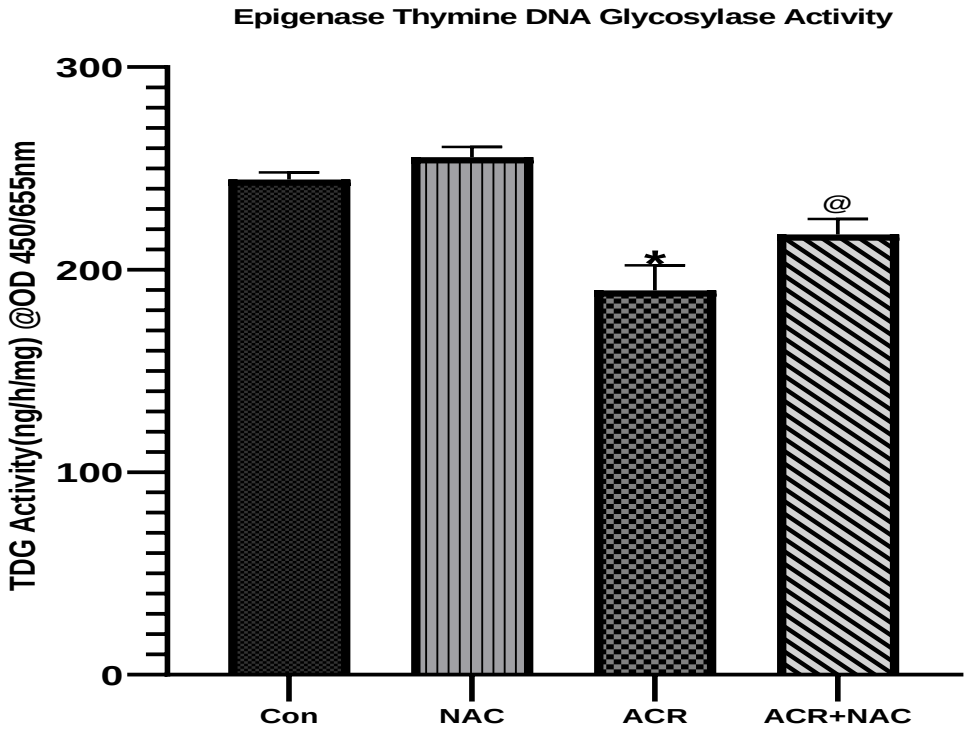
Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. Kidney tissue was used to extract nuclear protein (10µg) for 5mC Hydroxylase TET activity. Activity from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean ±SEM. (error bars) * $P \leq 0.05$ compared to Con, $^{@}P \leq 0.05$ compared to ACR. 5mC Hydroxylase TET activity showed significant (*$P \leq 0.0126$ and $^{@}P \leq 0.0055$).

Figure 4.7: Effects of Acrolein/ NAC Treatment on DNA Demethylase Activity



Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. Kidney tissue was used to extract nuclear protein (10µg) for demethylase activity. Activity from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean ±SEM. (error bars*)* *$P \leq 0.05$ compared to Con, $^{@}P \leq 0.05$ compared to ACR. Demethylase activity showed significant (*$P \leq 0.0234$ and $^{@}P \leq 0.0190$).

Figure 4.8: Effects of Acrolein/ NAC Treatment on Thymine DNA Glycosylase Activity



Sprague Dawley rats were treated with 2mg/kg/day of acrolein in the presence or absence of 600mg/kg/day of NAC. Kidney tissue was used to extract nuclear protein (10µg) for demethylase activity. Activity from control (Con) and acrolein (ACR) treated rats with or without NAC for 7 days (acrolein and NAC group) and 3 days pretreated of NAC following acrolein treatment for 7 days for ACR+NAC group. Values are expressed as mean $\pm$SEM. (error bars) *P $\leq$ 0.05 compared to Con, $^{@}$P $\leq$ 0.05 compared to ACR. TDG activity showed significant (*P $\leq$ 0.0265 and ns $^{@}$P $\leq$ 0.1149).

4.10 DISCUSSION

The objective of this study was to examine acrolein-induced epigenetic modification of DNA in rat kidney tissue. Humans exposure to several environmental pollutant acrolein, a very toxic compound, can be via inhalation, dermal contact, ingestion, and endogenously production of the compound in the body through lipid peroxidation process. Elevated level of acrolein in the body system has been indicated in variety of diseases such as neurological, inflammatory, and cardiovascular diseases, cancer etc. (Akshata et al., 2015). N-acetyl cysteine (NAC), through its antioxidant activity, can be a god candidate to inhibit acrolein toxicity and the subsequent deleterious effects.

The significance of DNA methylation is stressed by the increasing number of human diseases that are known to take place when DNA methylation is improperly recognized and/or sustained. Abnormal DNA methylation linked to amplified expression or the activity of DNMTs has been found in several diseases, particularly in cancer, in cardiovascular and respiratory diseases, metabolic and degenerative disorders. Inhibition of DNMTs may lead to demethylation and expression of silenced genes as well as a therapeutic solution (Santos et al., 2007).

Our previous studies with VSMC has shown acrolein capability to modify histones H3 which is known to play vital role in gene expression and transcription (unpublished data).

Furthermore, we observed that NAC has the ability to inhibit acrolein toxicity and the subsequent deleterious effect through prevention of histones H3 modification. The goal of the current study was to ascertain if acrolein induced toxicity was via DNA

methylation, and changes of methylation and demethylation enzymes. Rat models treated with 2mg/kg/day of acrolein for seven days in the presence and absence of 600mg/kg/day per body weight of NAC for 10 days were used to test our hypothesis.

4.11.1 Changes in animal weight

Treatment of rats with subtoxic level of acrolein for seven days did not result in any significant changes in the animal weight at the end of this study (Table 4.1).

4.11.2 Effect of Acrolein in Lipid Peroxidation

Many investigations have shown that free radicals are the major cause of oxidative stress. Increased oxidative stress contributes to lipid peroxidation. Lipid peroxidation is the process of oxidative degradation of lipids by free radicals which removes electrons from critical lipids in the cell membranes. At the end of the process, reactive aldehydes such as malondialdehyde and 4-hydroxynonenal are produced that can be measured for determination of changes in lipid peroxidation (Ron Shah et al., 2021). The aldehydes produced at the end of lipid peroxidation have reported to be important signaling intermediates in regulating important transcription factors and ultimately, resulting in modification of physiological functions (Baliga et al., 2013).

Lipid peroxidation has been implicated in different disease conditions such as cancer, atherosclerosis, acute lung injury, chronic alcohol exposure (Jie Wang, et al., 2021, Thiago C, et al., 2021). Our previous study with Vascular Smooth Muscle cells has shown that acrolein induced lipid peroxidation (Unpublished data) through the generation of free radical. To confirm that acrolein induces toxicity is via lipid peroxidation in rats

and to show whether NAC could reverse the toxicity, we measured level of lipid peroxidation in rats exposed to acrolein with or without NAC. Levels of lipid peroxidation products were measured in rat kidney tissue homogenate using ABCAM Lipid Peroxidation (MDA) Assay Kit. The result showed that lipid peroxidation level was increased by approximately 169% in acrolein treated rats compared with untreated group (Figure 4.1). Addition of NAC reduced lipid peroxidation by approximately 52% when compared with acrolein group. The increased in lipid peroxidation in acrolein treated group can be explained by the fact that acrolein contributes to increased level of free radicals as well as itself acting as a free radical and ultimately damaging polyunsaturated lipids in the cell membrane. Additionally, increased in free radical generation can damage endogenous antioxidant system resulting in depletion of the cellular antioxidant. Our data also showed that NAC impedes acrolein effect which may be through the enhancement of cellular antioxidant. Our results also agree with (Zahid Lepara et al., 2020) who carried out a study on serum MDA levels as a potential biomarker of cancer progression for patients with bladder cancer. The study included 90 patients among whom are tobacco smokers. Serum MDA level revealed a significantly increased in the distribution of tobacco smoker patients by 53.3% compared to 46% of control group (non-smokers). They concluded that MDA serum level might play a significant role as a biomarker in the diagnosis of bladder cancer.

In addition, research has laid emphasis on the importance of oxidative stress outcomes in cancer development and the function of antioxidants in the prevention and treatment (Valko M. et al., 2004).

4.11.3 Global DNA Methylation (5-mC)

DNA methylation is the most broadly studied epigenetic alteration where a methyl group is affixed to the segments of DNA. This fixing may result in either turn off of gene, suppress or inactivation by means of hindering the production of proteins from the altered gene. The outcome of modifications might result in an unusual gene transcription and expression which is known to cause genetic disorders, such as metabolic and degenerative disorders, cardiovascular and respiratory diseases, and cancer (Santos et al., 2007). The significance of 5-mC as an important epigenetic modulation in phenotype as well as gene expression has been acknowledged extensively. Some studies have reported that, a decrease in global 5-mC i.e., DNA Hypomethylation is probable instigated by methyl deficiency owing to a variety of environmental influences (e.g., acrolein) and has been projected as a molecular marker in numerous biological methods such as cancer (what is Epigenetics., 2021). Exposure to air pollutants has been reported to contribute to development of cardiovascular diseases. It has been hypothesized that these exposures contribute to changes in gene expression either directly or indirectly. For this reason, studying epigenetic changes has become an area of interest to explain some of the epigenetic modifications. To understand whether exposure to acrolein affects DNA, we measured global DNA methylation in the present or absence of NAC. Our result (Figure 4.2) demonstrated about 16% increase in 5-mC level in acrolein treated group when compared with untreated control. This could indicate acrolein has the capacity to induce toxicity via hypomethylation which agrees with Melanie Ehrlich et al., (2009) reporting that DNA hypomethylation was the initial epigenetic abnormality known in human tumor

development. Similarly, another study reported DNA methylation patterns vary significantly following smoke exposure and during exacerbations caused by bacterial infections in COPD progression. This group utilized in vitro models that mimics COPD and assessed the role of NF-kB and STAT3 methylation promoter. Human A549 cells were exposed to cigarette smoke extract (CSE) for 3 hours and also 3-hour exposure with a bacterial lipopolysaccharide prior to termination to CSE exposure. The results showed a significance hypomethylation of NF-kB mediated pathway genes linking it with their initiation of COPD exacerbation in study CSE model (Gagandeep Kaur et al., 2020). Addition of NAC resulted in about 16% increase in 5-mC level when compared to acrolein group. In addition, a study by Kanidta Sooklert et al., (2019) also revealed that NAC reverses the decrease in methylation status caused by engineered cold, silicon, and chitosan nano particles. Human embryonic kidney and human keratinocyte (HaCaT) cells were exposed to three separate types of ENPs: gold nanoparticles, silicon nanoparticles (SiNPs), and chitosan nanoparticles (CSNPs). The results indicated that the HaCaT cells exposed to SiNPs exhibited increased ROS levels, whereas HaCaT cells that were exposed to SiNPs and CSNPs experienced global as well as Alu hypomethylation, with no change in LINE-1 being observed in none of the cell line. The demethylation of Alu in HaCaT cells following exposure to SiNPs and CSNPs was inhibited when the cells were pretreated with NAC.

4.11.4 Global Hydroxymethylation (5-HmC)

Several investigators have confirmed the role of DNA methylation and hydroxymethylation as potential players in body response to air pollutants. 5-Hydroxymethyl cytosine is the product of TET oxidative breakdown of 5- methyl cytosine in the demethylation process. The biological importance of 5-hmC as an essential epigenetic variation in phenotype and gene expression has been recently identified. For instance, global decrease in 5-hmC level (DNA hypohydroxymethylation) has been displayed in just about all cancers and has been projected as a molecular marker and curative target in cancer (Gerd P Pfeifer et al., 2013). In the present study, to better understand the effects of environmental exposures to acrolein and the roles of epigenetic mechanism in acrolein-induced toxicity, we measure the level of global 5-HmC in rats treated with acrolein in the presence or absence of NAC. The collected data indicated up to about 22% decrease in 5-HmC level when compared with control (figure 4.3). Both markers of DNA methylation, 5-mC and 5-HmC, are products of DNMTs and TET enzyme activities respectively, have opposite functions. While 5-mC has been reported to result in suppression of gene expression, 5-HmC has been reported to cause activation and /or maintenance of gene expression. For that reason, in any epigenetic study, it is important to measure level of both these products to have a better understanding of epigenetic changes (Summary of results from Geneva World Health Organization burden of disease from ambient Air pollution for 2014). Swati Kadam et al., (2013) have also reported an association between carcinogenesis and loss of global 5hmC. In the same manner, a liquid chromatography-mass spectrometry, anti-5hmC antibody-based

immuno-dot blots and immunohistochemistry investigation has exhibited tumor-linked with global loss of 5hmC in cancers of the lung, brain, breast, liver, kidney, prostate, intestine, uterus, and melanoma (Jin SG et al., 2011). In our study, addition of NAC resulted in approximately 130% increase in 5-HmC level when compared with acrolein group, indicating NAC beneficial effects might be through upregulating 5-HmC activity.

4.11.5 DNA Damage Quantification

Oxidative stress is one of the common mechanisms proposed for acrolein-induced toxicity (Moghete et al., 2015). 8-OHdG, a sensitive biomarker of oxidative DNA damage is produced from hydroxyl radicals generated from the derivative of deoxyguanosine. Numerous studies have implicated 8-OHdG to be vital not only due to its abundance but its mutagenic potential via G- transversion upon duplication of DNA. 8-OHdG is known to play a significance role in epigenetic regulation of gene initiation/suppression by constraining the binding affinity of MBD protein to the CpG site of DNA. Also, 8-OHdG is a crucial marker for measurement of oxidative DNA damage in humans after exposure to cancer agents such as tobacco smoke, asbestos fibers, heavy metals, and polycyclic aromatic hydrocarbon as well as risk factor for many diseases wherein cancer is included (Athanasios et al., 2009).

Various investigators have reported changes in the level of 8-OHdG is linked to the prognosis of cancer but the precise biomarker for diagnosis of human cancer is still unclear (Xiangcheng Qing et al., 2019). Biological studies have to consider exposure

level and analytical methods of measuring 8-OHdG when drawing conclusion as it could present an inconsistent effect (Sajous et al., 2008).

In the present study, we wanted to assess the relation between exposure to acrolein and the occurrence of DNA damage by measuring biomarkers effect, 8-OHdG. Furthermore, we were interested to see if addition of NAC can prevent the damage. The results (Figure 4.4) showed that the group treated with acrolein had approximately 18% decrease in 8-OHdG level when compared with untreated control. This could be interpreted as acrolein inducing DNA damage through oxidative stress. Our results are in agreement with Deyao et al., (2019) that low 8-OHdG expression is related with the prognosis of cancer. The addition of NAC showed about 167% increase in 8-OHdG level when compared with acrolein group which could indicate the ability of NAC to hinder the induced toxicity caused by acrolein and reversing DNA damage (Sooklert et al., 2019).

4.11.6 DNA Methyltransferase Enzyme (DNMT) Activity

DNA Methyl transferase enzymes also known as DNMTs are group of enzymes that control the addition of methyl groups from S-adenosyl- L-methionine (SAM) to the fifth carbon position of cytosine rich CpG island of the DNA. The aberrant methyl group addition by DNMTs is the sole cause of DNA methylation (de novo or maintenance) which has been associated to different abnormal DNA function that results in the epigenetic modification of gene transcription and expression. Consequently, these modifications have been shown in different studies to be the onset of various chronic diseases such as different types of cancer and cardiovascular disease. (Jiayu Zhang et al.,

2020). Studies has also stated that unusual DNMT activities impinge on tumor-related factors for instance genomic instability plus hypermethylated suppressor which increase the growth of tumors, exacerbate the diagnosis for patients, and greatly escalate the complexity of cancer treatment. (Cheng Yang et al., 2020). To further, elucidate the mechanism of epigenetic changes in rats exposed to acrolein, we investigated changes in DNMT enzymes and whether NAC could hinder the effect of acrolein and thus, reversing DNA methylation. Our observation (Figure 4.5) showed that rats treated with acrolein expressed a 30% increase in DNMT activity when compared with untreated control. Addition of NAC further increased DNMT activity by 32%. The activity of DNMT is highly regulated. It has been reported that interactions with different molecules, post translational modifications, alternative silencing and gene duplication or gene loss are the main regulatory mechanisms of DNMT activity. Considering the high activity of acrolein and its action in inducing generation of free radicals, it is not surprising to see the observed changes in our study. Mutation of DNMT has been reported in association of several diseases, including several cancers. It is possible that acrolein is affecting the balance between changes that contribute to higher DNA methylation and removal of the methyl group. It is possible that modification of expression of enzymes such as DNMT and TET as well as contribution of oxidation is leading to global hypomethylation by acrolein and the consequence toxicity. This observation agrees with a study done by Pankaj et al., (2014) disclosing the DNMT activity and the role of N-methyl-D-aspartate receptor-1 (NMDAR1) in epigenetic mechanisms of heart failure, using cardiomyocytes during hyperhomocysteinemia (HHcy) utilizing HL-Cells treated with 4 different

concentrations of Hcy. The result showed that the group with high level of Hcy showed an increase in DNMT activity which echoed a global methylation. Although we were anticipating a decrease in DNMT activity upon addition of NAC, to our surprise, this group showed further increase in DNMT activity. Furthermore, investigation will be conducted to better understand the observation.

4.11.7 5mC Hydroxylase TET Activity

Cytosine methylation and consequent oxidation process has been the bedrock of epigenetic modification in human and animal genomes. 5mC is a natural cytosine nucleotide around the DNA which has been modified through the addition of methyl group at the fifth carbon. This addition is very important, it has been believed that 5mC is the DNA fifth base. 5mC plays a crucial role in transcription process as a gene repressor. In promoters, 5mC is linked to steady, long-term transcriptional silencer. This could happen either through opposing positive transcription factors or by enhancing the attachment of negative ones. The aberrations have mainly been indicated in diverse prognosis of diseases which cancer development is one of them. Ten eleven translocation enzymes have been discovered to be the bridge between 5-mC methylation and demethylation through the oxidative breakdown of 5-methyl cytosine to 5-hydroxycytosine and further regulates the conversion of 5hmC to 5-formylcytosine(5fC) and then 5fC to 5-carboxylcytosine (5caC) forming the basis of cytosine methylation reversal. It also play a vital role in regulating epigenetic alterations (Jungeun An et al., 2017).

Research has shown that genomic 5-HmC levels correlate with TET hydroxylase activity. In this study, we evaluated the effect of acrolein on the role of TET enzymes in DNA methylation/demethylation and the effect of NAC on this process. 5-mC Hydroxylase TET Activity was measured with kidney tissue from different groups for measurement of total activity/inhibition of cytosine oxygenase TET enzymes in nuclear extracts. Results indicated that the treatment of rats with acrolein resulted in approximately 67% decrease in 5mC Hydroxylase TET activity when compared with untreated control. Addition of NAC resulted in approximately 28% increase in 5mC Hydroxylase TET activity (Figure 4.6). TET loss of function has been reported in various cancers and restoration of TET expression and /or function has been suggested as a possible approach for reversing the clinical impact in those situations. Our results correlate with the effectiveness of TET enzymes in DNA demethylation process reported by Kasper and Kristian (2016). It also agrees with Xirong Lu et al., (2020) who carried a study to show the potency of TET3 in the inhibition of programmed cell death protein 1 (PD-1) affected by oleanolic acid (OA) promoting DNA methylation in gastric cancer cells (MKN-45). The result showed a reduced expression of IL-1β-treated MKN-45 cells enhanced with OA and overexpression of TET3 restored OA-reduced PD-L1 expression.

Aberrant DNA methylation has been reported in different cancer types (Bhusari et al., 2011) as well as autoimmune diseases, metabolic disorders, neurological disorders, and aging (Zelin Jin and Yun Liu., 2018). As an ubiquitous environmental pollutant as well as the high activity of acrolein and functioning like a free radical, it is not surprising to see observed effect by acrolein on 5mc Hydroxylase TET enzyme. There are reports of

studies reporting low methylation in asthmatic children (Somineni et al., 2016). We are suggesting that oxidative stress associated with exposure to acrolein has contributed to the modification of TET enzymes expression which ultimately affects 5-HmC level. Other investigators have also reported that exposure to toxicant in cigarette smoke (benzene and benzene metabolites) contributes to global increase in 5HmC levels and TET1 activity (Coulter et al., 2013) and have suggested that TET1 plays a vital role in responding to pollutants (Bollati et al., 2007).

4.11.8 DNA Demethylase Activity

DNA demethylases are enzymes that eliminate methyl groups from DNA, proteins (especially histones) and other cellular molecules. DNA demethylase enzymes are vital in epigenetic modulation mechanisms. Demethylases modify transcriptional regulation of the genome by regulating the methylation levels that takes place in DNA and histones and therefore, control chromatin structure at specific gene sites within the organisms. Biological importance of DNA demethylases is its effective mediation in DNA demethylation which is an essential epigenetic process involved in maintaining normal gene expression. (Weiwen et al., 2020). To further evaluate the underlying mechanism of epigenetic changes in acrolein treated rats, we measured DNA demethylase activity. Our results indicated that exposure to acrolein resulted in about 14% decrease in demethylase activity when compared to control group. Addition of NAC prevented the effect of acrolein on DNA demethylase activity (Figure 4.8). Our findings is suggesting that increased oxidative stress as the result of exposure to acrolein is resulting in an imbalance between DNA methylation and demethylation through effecting enzymes such as DNA

demethylase. Imbalance of DNA methylation and demethylation have been reported to be linked to pathological conditions such as cancer, asthma, and diabetes (Ji H et al., 2016; Zhang X et al., 2017; Spearman et al., 2018).

Ryu et al., (2019) have reported that exposure to environmental pollutant PM 2.5, resulted in a decrease in DNA methyltransferase (DNMT) expression and an increase in DNA demethylase expression, and ultimately causing hypomethylation of p16INK4A promoter region.

4.11.9 Thymine DNA Glycosylase Activity (TDG)

The significance of Thymine DNA glycosylase in DNA demethylation, repair, and regulation of gene transcription has been established. TDG is known to participate in the demethylation process of 5- methyl cytosine after numerous oxidations by TET enzymes. TDG converts the 5-carboxyl cytosine to abasic cytosine that is next repaired to the original status by base excision repair (Kohli RM et al., 2013; Charles E et al., 2021). In this experiment, we wanted to assess the effect of acrolein on TDG enzyme in rats and the consequence epigenetic changes. Our results showed that there was an approximately 22% decrease in TDG activity in the group treated with acrolein when compared with untreated control and addition of NAC contributed to approximately about 15% increase in TDG activity (Figure 4.9). This result indicates acrolein prevents demethylation function of TDG and ultimately inducing methylation. These findings suggest that TDG plays critical role in prevention of toxic effect of environmental pollutants by preventing epigenetic modifications caused by these pollutants functions specific to cancer cells that

make it highly suitable anti-melanoma drug test (Pietro et al., 2019; Mancuso et al., 2019).

This result also acceded with an examination of TDG in reversing chromatin condensation DNA repair and demethylation. This was done by assembling 12-mer nucleosome arrays containing fluorescent donor and acceptor dyes that were placed at locations that enables nucleosome stacking interactions, and the complete compaction of the array specifically, the formation of 30 nm chromatin fibers, which was checked by FRET (Forster resonance energy transfer). 2 mM of magnesium ion was added to compact the nucleosome arrays. Addition of 200nM of TDG, reduced Fret efficiency by approximately 20% suggesting that TDG caused decompaction of the nucleosome arrays (Deckard et al., 2021) they confirmed their result by observed TF FOXA1, which is recognized to actively introduce chromatin decompaction and boost DNA accessibility (Cirillo et al., 2002). This shows that TDG have vital biological duty since its involvement in DNA demethylation, chromatin remodeling, and transcriptional control.

CHAPTER 5

SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary

Exposure to environmental pollutants have been reported to contribute and/ or exacerbate disease conditions, which makes it important to understand the mechanism of toxicity of individual pollutants. Acrolein is a common environmental pollutant that humans are exposed to through a variety of ways. In the present study, we investigated epigenetic changes in rats exposed to sub-lethal dose of acrolein in the presence or absence of NAC, a natural antioxidant. Exposure of animals to a sub-toxic dose of acrolein 2mg/kg/day for a short period of time (7 days) did not cause any apparent physiological change. As a result, there was no significant difference between the weight changes of animals in treated groups compared to the control.

It is well understood that changes at molecular and cellular level way exceed the time of the apparent physiological damage. Therefore, in this study, our goal was to establish epigenetic changes as a result of exposure to acrolein and the possible mechanisms involved at low dose exposure of acrolein.

In the present study, we are reporting high level of lipid peroxidation in acrolein-treated rats, which was alleviated after the addition of NAC. Furthermore, measurement of global methylation indicated a 16% increase in 5-mC level in acrolein treated group compared with control group. Generation of free radicals have reported to contribute to

epigenetic changes and inflammation in different diseases. Increased free radicals can affect gene expression through influencing epigenetic mechanisms such as DNA and Histone Methylation. This can be the result of destruction of S-adenosyl methionine which is important in the control of gene expression by DNA and histone methyltransferases. Glutathione (GSH) which has been reported to be diminished as a result of high generation of free radical species, might be an intermediate player in this process. Additionally, we attempted to shade lights on the possible mechanisms that are contributing to epigenetic changes in acrolein treated rats. We investigated changes in methylation /demethylation enzymes, reporting a decrease in TET enzymes in acrolein treated group and a significant increase after addition of NAC (Figures 4.4 and 4.5). The result highlights TET enzymes as the bridge between 5-mC methylation and demethylation through the oxidative breakdown of 5-methyl cytosine to 5-carboxylcytosine (5caC) forming the basis of cytosine methylation reversal as well as play a vital role in regulating epigenetic alterations. Furthermore, the steady reduction was observed in activity of the demethylation enzyme DNA demethylase (Figure 4.6) which is consistent with acrolein exerting toxicity and initiation of DNA methylation as well as the potency of NAC antioxidant function. The same effect was seen in Thymine DNA Glycosylase activity (Figure 4.5).

The present study also demonstrated that acrolein induced DNA methylation by altering the activity of DNA methyltransferase (Figure 4.6) which is the enzyme responsible for the addition of methyl group to the fifth carbon atom of cytosine in the CpG island of DNA. This addition has been associated with different abnormal DNA function that

results in the epigenetic modification of gene transcription and expression. The result revealed a significant increase in DNMT activity when compared to untreated control group agreeing with the goal of the study of acrolein induced toxicity via DNA methylation. Furthermore, we evaluated level of global 5-HmC in rats treated with acrolein in the presence or absence of NAC. The collected data indicated a significant decrease in 5-HmC level when compared with control group.

Both markers of DNA methylation, 5mC and 5Hmc, are products of DNMTs and TET enzyme activities but they have opposite functions. While 5mC has been reported to result in suppression of gene expression, 5Hmc has been reported to cause activation and/or maintenance of gene expression.

In our study, we observed a significance decrease in 5HmC level in acrolein-treated rats. Similar reduction of 5HmC level has been reported in just about all types of cancers and has been projected as a molecular marker and curative target in cancer. Finally, we quantified DNA damage due to acrolein toxicity by measuring 8-OHdG level (the oxidative biomarker for DNA damage) (figure 4.9). We observed a decrease in 8-OHdG in acrolein group compared to untreated control which could indicate acrolein inducing DNA damage through oxidative stress.

5.2 Conclusion

Based on the present data, we are concluding that epigenetic changes observed in acrolein-treated rats are the results of increased generation of ROS, which contribute to increase oxidation level, alteration of TET enzymes and their products, and increased DNA methylation and subsequent DNA damage.

There are important implications of our findings. Our data suggest that acrolein exposure may have great effect on triggering epigenetic changes by targeting key regulating enzymes and thus leading to the development of inflammation. Furthermore, we are proposing that addition of NAC can be an important approach in preventing/reducing the toxic effect of acrolein.

5.3 Recommendation

Interleukins are involved in the initiation of pro-inflammatory responses in the body during invasion of toxins or injury state. ROS have been reported to affect pro-inflammatory cytokines such as TNF-a, IL-1 and IL-8 which are critical in inflammatory responses. Since acrolein contribute to increased changes in ROS and itself acts like a free radical, we recommend more investigation to be carried out to investigate the link between changes in TNF-a, IL-1 and IL-8 and epigenetic changes in acrolein-induced toxicity. Furthermore, investigating individual members of TET enzyme family and DNMTs and their various connection with acrolein is important. Finally, Further confirmation of presented data using different methods can also add valuable information in the confirmation of these results.

APPENDIX

Future Studies

Based on the data obtained from this study and the observed effects on changes in the activity of important enzymes involved in epigenetic modification, it is important to confirm/link changes in the expression of proteins of these enzymes and their activity. Therefore, we will perform western blot analysis to evaluate the effect of acrolein/NAC on methylation and demethylation enzymes (TET1, DNMT 1, and DNMT3A) protein expressions and check if it correlates with the enzyme activities that we have already reported. The expression of these proteins will confirm obtained data and enable us to make a better conclusion on changes found in epigenetic enzymes due to acrolein treatment. We will also confirm NAC antioxidant function on reversing or reducing acrolein induced DNA methylation through changes in demethylation enzymes.

The analysis will be carried out using the following procedure:

An average of 100g of the kidney tissue from each group was homogenized in RIPA lyses buffer (Santa Cruz Biotechnology) and protease inhibitor cocktail (HALT; Thermo Scientific) using a Tissue- Lyser (Qiagen). Homogenates will be centrifuged at 4°**C** at 1200 rpm for 20 minutes. The supernatants will be collected and used for the biochemical analysis. Protein concentrations will be determined using BCA protein Assay kit and reading was taken at 562nm. A total of 30µl of protein from kidney homogenate will electrophoresed on 8% polyacrylamide gels. After two hours of electrophoresis (@ 50v first and then @ 150v), gels will be transferred overnight to PVDF membrane (Bio-Rad

Laboratories Inc. CA) @ 25v x 25max 100w. Blots (Membranes) will be probed (incubated) overnight with corresponding primary antibodies.

For DNMT1 antibody dilution was at 1:500 dilution, (Epigentek), DNMT3A at 1:500 dilutions, (Epigentek),), and TET1antibody at 1:1000 dilution (Epigentek), After primary incubation and washing off of primary antibody, respective secondary antibodies (HRP-Goat Anti-Rabbit lgG and anti-Mouse) will be added to membranes at 1:10000 dilutions and incubated for one hour. After incubation and washing, Odyssey Substrate detection system from Licor (Lincoln, NE) with enhanced infrared fluorescence will be used to visualize Immuno-complexes. The intensity of the bands will be scanned and quantified using Odyssey Classic Scanner (Lincoln, NE). Buffer for 30 minutes and probed with GAPDH antibody (1:5000) as house-keeping protein and also scanned for protein expression. Quantitative measurements will be performed with GraphPad Prism 5 software.

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