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INVESTIGATION OF THE OXIDANT-ANTIOXIDANT SYSTEM IN NADPH5 KNOCK-OUT RABBITS AS A RESPONSE TO CHOLESTEROL RICH DIET

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ABBREVIATIONS

Abbreviation	Full Form
ROS	Reactive Oxygen Species
CVDs	Cardiovascular Diseases
NOX	NADPH Oxidase
NOX5	NADPH Oxidase 5
SOD	Superoxide Dismutase
CAT	Catalase
PRDX	Peroxiredoxin
qPCR	Quantitative Real-Time Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
ATP	Adenosine Triphosphate
LDL	Low-Density Lipoprotein
ox-LDL	Oxidized Low-Density Lipoprotein
BBB	Blood-Brain Barrier
APP	Amyloid Precursor Protein
Aβ	Amyloid-beta
MRI	Magnetic Resonance Imaging
NAA/Cr	N-acetylaspartate to Creatine Ratio
Glu/Cr	Glutamate to Creatine Ratio
AD	Alzheimer's Disease
HPRT	Hypoxanthine Phosphoribosyltransferase

YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta
WT	Wild-Type
KO	Knock-Out
Tm	Melting Temperature
RIN	RNA Integrity Number
UV	Ultraviolet
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
mRNA	Messenger RNA
EDTA	Ethylenediaminetetraacetic Acid
SDS	Sodium Dodecyl Sulfate
Ct	Cycle Threshold
NRF2	Nuclear Factor Erythroid 2–Related Factor 2
KEAP1	Kelch-Like ECH-Associated Protein 1
ALS	Amyotrophic Lateral Sclerosis
GSH	Glutathione
GPX	Glutathione Peroxidase
IMDDHH	Immunodeficiency, Developmental Delay, and Hyperhomocysteinemia

ABSTRACT

Cardiovascular diseases (CVDs) represent a serious global health burden, with oxidative stress playing a crucial role in their pathogenesis. Oxidative stress occurs due to imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses which is influenced by genetic, epigenetic and environmental factors including dietary components such as the intake of cholesterol. This thesis evaluates the role of *NOX5*, a member of the NADPH oxidase family, on gene expression alterations in the brain in the context with oxidative stress in genetically modified *NOX5* knockout rabbits under dietary stress, in the form of a cholesterol-rich diet. My findings highlight the role of oxidative stress caused by a cholesterol-rich diet in the rabbit brain and point out the importance of *NOX5*. Using *NOX5* knockout rabbits on varied dietary regimens, this study investigates the association between genetic modifications, dietary factory, and cardiovascular health. The methodology comprises of a controlled experimental design with standard and cholesterol rich diets given to both wild-type and *NOX5*-deficient rabbits. Gene expression was analyzed using a Quantitative Real-Time PCR (qPCR) with a focus on significant oxidative stress-related genes. The hypothetical findings of this thesis indicate that dietary cholesterol significantly impacts the expression of essential antioxidant genes and might alter the expression of *NOX5*. *NOX5* knockout rabbits had varied antioxidant responses under various dietary conditions, highlighting the complex association between dietary influences and genetic background on oxidative pathways. The implications of these findings expand to understanding the molecular pathways through which *NOX5* and cholesterol interact and how this is manifested in the brain. By finding possible therapeutic targets within the oxidant-antioxidant system that may lower cardiovascular risk associated with dietary cholesterol, this study broadens our knowledge and opens the door for future research into the role of diet and genes in cardiovascular pathology.

Keywords: *Oxidative stress, Reactive oxygen species, NOX5, NADPH oxidase, Cholesterol, Dietary influence, Antioxidants, Knockout rabbit model*

1. INTRODUCTION

Cardiovascular diseases (CVDs) remain the greatest threat to global health and are one of the major global causes of morbidity and mortality. In the pathogenesis of CVD oxidative stress plays a crucial role (Berger et al., 2015). Oxidative stress occurs due to the imbalance between ROS production and the ability of the body to neutralize their harmful effects by antioxidant defenses. While ROS is necessary for physiological processes like pathogen defense and cell signaling, an excess of ROS can also result in cellular damage (Berger et al., 2015). Thus, oxidative stress is considered as the core of pathogenesis of a variety of CVDs due to an imbalance between ROS and antioxidant defense systems (Berger et al., 2015).

The NADPH oxidase family of enzymes appears as a crucial source of vascular ROS, with its members being widely researched for their functions in controlling oxidative stress and thus impacting cardiovascular health (Touyz et al., 2019). Among the NOX family members, *NOX5* has emerged as a significant contributor to vascular oxidative stress due to its calcium-dependent activation and ability to produce ROS. However, dietary factors, especially cholesterol intake have been shown to modulate *NOX5* expression and activity, suggesting a link between diet, oxidative stress, and cardiovascular health (Touyz et al., 2019). However, the exact mechanisms by which *NOX5* and dietary cholesterol interact to influence CVD development remain incompletely understood.

This thesis is based upon the central hypothesis that knockout of *NOX5*, in the presence of a high-cholesterol diet, will exacerbate atherosclerosis development in a rabbit model through mechanisms involving dysregulated ROS production and impairment in antioxidant defense systems. Therefore, *NOX5* is also postulated to be bivalent in cardiovascular health, not only by being a large provider of ROS but also by serving as a homeostatic regulator for gene expression of antioxidants. In this regard, the research would be expected to depict that, under the stressor of a cholesterol rich diet, *NOX5* deficiency results in the alteration of important antioxidant gene expressions such as *SOD3*, *CAT*, and *PRDX*, thus increasing susceptibility to oxidative stress and facilitating atherogenesis. By investigating these mechanisms in *NOX5* KO rabbits, this thesis aims to contribute valuable knowledge on the pathophysiological roles of ROS and NADPH oxidases in cardiovascular diseases and unveiling new possible pathways for therapeutic intervention.

Aim and Objectives

The aim of this thesis is to investigate the role of *NOX5* in the context of oxidative stress and cardiovascular health, with a particular focus on how dietary cholesterol influences *NOX5* expression and activity, and the subsequent effects on oxidative stress markers and CVD development. The specific objectives of this study are to:

- Assess the impact of dietary cholesterol on *NOX5* expression and activity in both wild-type and *NOX5*-deficient (*NOX5* -/-) rabbit models.

- Evaluate the effects of *NOX5* deficiency on the expression of key antioxidant genes (*SOD3, CAT, PRDX*) under standard and cholesterol rich diet conditions.

- Investigate the relationship between *NOX5* activity, dietary cholesterol, and the development of atherosclerosis, by examining markers of oxidative stress and atherosclerotic lesion formation in rabbit models.

Through these objectives, this thesis aims to contribute to the broader understanding of correlation between genetic factors and dietary influences in the pathogenesis of CVDs, highlighting the potential of *NOX5* and dietary modifications as targets for therapeutic intervention.

2. LITERATURE REVIEW

2.1 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen that are generated in the body through various physiological processes (Apel & Hirt, 2004). These molecules play an important role in cell signaling and homeostasis. However, oxidative stress can occur due to the imbalance between ROS generation and antioxidant defense mechanisms. Oxidative stress could damage lipids, proteins, and DNA which may lead to diverse human diseases (Alkadi, 2020).

ROS can be generated via various cellular mechanisms as well as external factors. According to Alkadi (2018) some major systems within the body produce free radicals such as a subset of ROS (2018) (Figure 1.). These reactive molecules are essential for biological functions like cell signaling, homeostasis and regulatory response to immune activities. Nevertheless, excessive generation of ROS leads to oxidative distress where there is disturbance in the balance between antioxidants and free radicals including ROS (Alkadi, 2018).

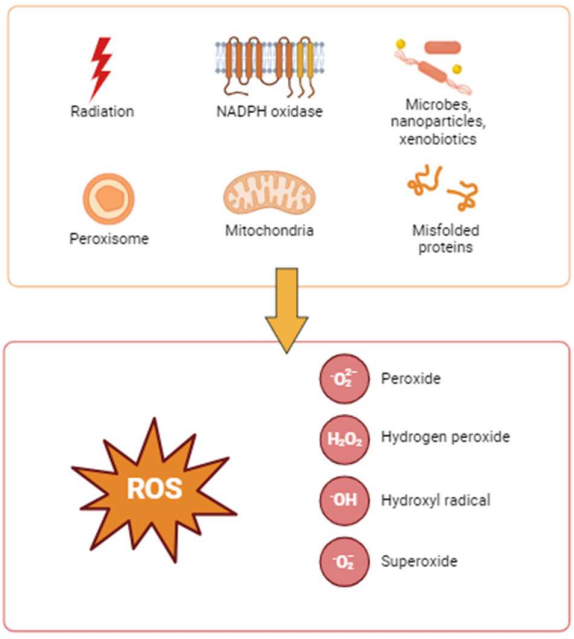

Figure 1: Sources of Reactive Oxygen Species (ROS) Generation

The prime sources of ROS in biological systems are the mitochondrial electron transport chain, oxidases, oxygenase and peroxidases as well as external sources such as environmental pollutants, UV radiation and cigarette smoke (Alkadi 2018). ATP-generating process in mitochondria leads to formation of ROS as by-products from the electron transport chain. In addition to that, NADPH oxidase enzymes also contribute to the production of ROS during immune responses against infections. Another research carried out by (Apel & Hirt 2004) shows that oxidative phosphorylation within the mitochondrial metabolism result in generation of ROSs also following various stimuli like xenobiotics and cytokines. ROS acts as signaling molecules for cell proliferation, differentiation or apoptosis (Apel & Hirt 2004).

Despite being necessary for normal cellular function, excessive levels of ROS can result into oxidative stress which results into damage on DNAs proteins and lipids; thus becoming connected with pathologies like cancer, cardiovascular diseases or even neurodegenerative diseases (Apel & Hirt 2004). Also, ROS contributes to tissue damage and organ dysfunction in conditions such as sepsis and pulmonary fibrosis (Apel & Hirt, 2004). According to Tenkorang et al., 2018, the degree of harmfulness or beneficialness of ROS is determined by their concentrations in the body. They play a role in regulating various cell functions like proliferation, differentiation and survival. Dikalov et al., (2013) and Rijk et al., (2022) claim that hypertension and aging-related processes have a relationship with ROS while Taucher et al. (2022) argue that an imbalance between production of ROS and the capacity for antioxidant scavenging can lead to disorders associated with oxidative stress like periodontal diseases.

2.1.1 Oxidative stress and atherosclerosis

Oxidative stress is a central factor in the pathogenesis of atherosclerosis, which is an inflammatory disease affecting medium and large arteries (Kattoor et al., 2017). Oxidative stress occurs due to imbalanced generation of ROS with the body's antioxidant system leading to vascular injury and atherogenesis (Kattoor et al., 2017). Oxidized low-density lipoprotein (ox-LDL) plays an important role in atherosclerosis by stimulating ROS generation in various cell types involved in the disease process. The association of LOX-1 scavenger receptor by ox-LDL results in foam cell formation, which is one of the first signs of early atherosclerotic lesions (Figure 2) (Kattoor et al., 2017). A number of endogenous ROS-producing systems are implicated in this condition including NADPH oxidase, xanthine oxidase and mitochondrial

enzymes that emphasize the significance of targeting oxidative stress pathways when designing therapeutic strategies for atherosclerosis (Kattoor et al., 2017). Traditional antioxidant supplements have shown limited effectiveness within clinical trials; ongoing novel approaches currently being explored include selective targeting oxidative stress in atherosclerosis through mitochondrial ROS scavengers, nanotechnologies-based drug delivery systems, gene therapies as well as anti-miRNAs (Kattoor et al., 2017).

Therapeutic interventions that modulate ROS generation and enhance antioxidant systems, such as aspirin, statins, and renin-angiotensin system inhibitors, demonstrate pleiotropic antioxidative effects in combating atherosclerosis (Kattoor et al., 2017). The research highlights the significance of identifying novel therapeutic modalities to selectively target oxidative stress in atherosclerosis, emphasizing the critical role of oxidative stress in the development and progression of this chronic inflammatory disease.

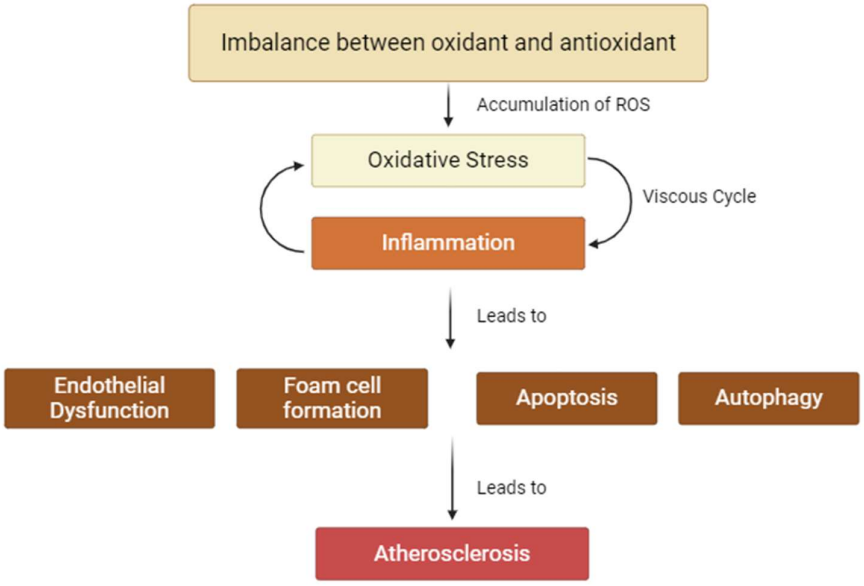

Figure 2: Oxidative Stress in Atherosclerosis

2.2 NADPH oxidase

Reactive oxygen species (ROS) play a crucial role in the pathogenesis of CVDs where NADPH oxidases act as the primary sources of oxidative stresses in the cardiovascular system (Cave et al., 2006). The fact that NOX family is distinct from other enzymatic systems due to its primary role in ROS production rather than a byproduct differentiates it as an important enzymatic source of ROS (Cave et al., 2006). NOX enzymes are special among other enzymes for causing cardiovascular pathologies by generating superoxide and hydrogen peroxide, which in turn induce ROS release from other enzymatic systems in a cascade (Cave et al., 2006). In cases of hypertension, atherosclerosis and heart failures, excessive production of ROS results in endothelial dysfunction, inflammation within vascular walls, and damage to structural heart tissues (Cave et al., 2006). NOX-derived ROS enhances secondary oxidase systems through networking with other oxidase systems thus escalating oxidative stress rates and maintaining continued synthesis of free radicals (Cave et al., 2006). It is this recurrent situation that accounts for high levels of oxidative stress often observed in CVD, worsening tissue damage and disease progression (Cave et al., 2006)

It is crucial to understand this complicated network for unraveling how oxidative stress affects the cardiovascular pathologies (Cave et al., 2006). Different cardiovascular circumstances are regulated by certain NOX isoforms, such as hypertension being associated with *NOX1* and *NOX2* while diabetes vascular complications with *NOX4* (Rivera et al., 2010). It is notable that targeted inhibition of these specific NOX enzymes may form a basis of CVDs therapy indicating the significance of ROS-producing mechanisms targeting precision (Rivera et al., 2010). The therapeutic implications of targeting NOX systems and their interactions with other oxidase systems are discussed, advocating for a deeper understanding of NOX-mediated oxidative stress mechanisms to develop effective therapies for preventing or treating CVDs (Cave et al., 2006).

Further research into the pathobiology of NOX enzymes and their interactions within the broader ROS-producing network is important for identifying novel therapeutic targets and strategies (Cave et al., 2006). This study has indicated that NADPH oxidase within high cholesterol diet-induced changes in gene expression in rabbit model lacking gene which encodes for *NOX5* results in development of artery hardening due to NADPH oxidase. Regardless of its low expression in certain organs, *NOX5* can impact atherosclerosis

development. This emphasizes the importance of NOX enzymes, including *NOX5,* in the pathogenesis of cardiovascular diseases, emphasizing the need for targeted interventions to mitigate oxidative stress and its detrimental effects on cardiovascular health.

2.2.1 Basic role and physiology of NADPH Oxidase

NADPH oxidases (NOX) are essential enzymes in mammalian cells responsible for generating reactive oxygen species (ROS), which have been redefined as crucial signaling molecules rather than mere metabolic by-products (Dikalov, 2011). The NOX family consists of seven distinct isoforms, each with unique expression patterns, regulatory mechanisms, and functional roles in various tissues (Dikalov, 2011). While *NOX1-3* activation mechanisms depend on cytoplasmic subunits, *NOX4* exhibits constitutive activity, mainly producing hydrogen peroxide (H2O2) (Dikalov, 2011). *NOX5's* activation is calcium-dependent, and *DUOX1* and *DUOX2* have specialized functions in thyroid hormone biosynthesis, highlighting the isoform-specific roles in physiological and pathological processes (Dikalov, 2011). Genetic models, particularly studies involving Nox-deficient mice, have been crucial in understanding the physiological functions of NOX enzymes and their implications in human diseases. These models have emphasized the significant roles of *NOX2* in immune defense and *DUOX2* in thyroid hormone synthesis, illustrating the delicate balance between the beneficial signaling roles of ROS and their potential for cellular damage (Dikalov, 2011).

NOX-derived ROS has a dual role in cellular signaling, influencing cell proliferation, differentiation, and survival by affecting redox-sensitive proteins (Dikalov, 2011). These ROS impact critical signaling pathways, affecting cell growth, survival, metabolism, and immune responses, highlighting ROS as both signaling molecules and potential sources of cellular distress. The development of NOX inhibitors, evolving from non-specific to more targeted molecules, shows promise in treating diseases associated with excessive ROS production (Dikalov, 2011). The complex regulation and functional redundancy of NOX isoforms however pose challenges in developing specific inhibitors, necessitating further research to effectively harness the therapeutic potential of targeting NOX enzymes (Dikalov, 2011).

Study performed by (Schulze & Lee, 2005) describes that atherosclerosis is a complex disorder influenced by disturbances in lipid metabolism and inflammatory processes. Oxidative stress plays a significant role in the development of atherosclerosis (Schulze & Lee, 2005).

NADPH oxidases, such as *NOX1, NOX2, NOX4,* and *NOX5*, are crucial in generating ROS during atherogenesis (Schulze & Lee, 2005). *NOX1* is linked to hypertension and vascular pathologies, while *NOX2* affects endothelial functions and vascular smooth muscle cells. *NOX4*, known for producing hydrogen peroxide, exhibits atheroprotective effects by influencing vascular remodeling and inflammation (Schulze & Lee, 2005). *NOX5* is a significant source of ROS in atherosclerotic lesions (Figure 3) (Schulze & Lee, 2005). Another study performed by (Bedard & Krause, 2007) indicates that *NOX1*, predominantly found in vascular smooth muscle cells, contributes to vascular remodeling and atherosclerosis through cell proliferation and migration, regulated by growth factors and cytokines, *NOX2*, widely expressed in endothelial cells and phagocytes, plays a significant role in endothelial dysfunction, inflammation, atherosclerosis, and ischemia-reperfusion injury (Bedard & Krause, 2007). *NOX4*, with a more ubiquitous expression pattern, is involved in cell differentiation and contributes to fibrosis in cardiac and vascular injury models (Bedard & Krause, 2007).

NOX5 was discovered in 2001 by Lambeth (Cheng et al., 2001) and Krause (Banfi et al., 2001) laboratories. However, *NOX5* is less studied due to its absence in rodent models, implicated in calcium signaling pathways and vascular tone regulation (Bedard & Krause, 2007).

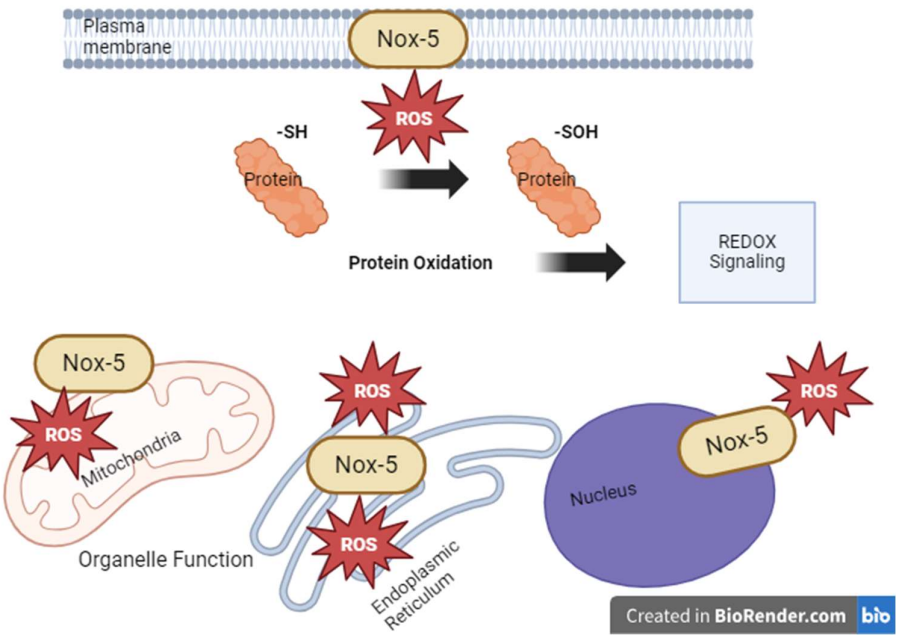

Figure 3: The role of *NOX5* in generating ROS and its effects on protein oxidation and redox signaling within different cellular compartments like mitochondria, plasma membrane, endoplasmic reticulum, and nucleus.

2.2.2 NADPH oxidase role in pathology

The role of NADPH oxidase (NOX) enzymes in the development and progression of heart and cardiovascular diseases is increasingly recognized due to their integral involvement in oxidative stress and inflammatory processes. A comprehensive review by Poznyak et al. (2019) identifies NADPH oxidases as key contributors to atherosclerosis, highlighting their role in matrix remodeling, modifications of low-density lipoprotein (LDL) particles towards a more atherogenic form, and the generation of oxidative stress. These enzymes, by facilitating these processes, emerge as potential targets for therapeutic intervention, suggesting that modulating their activity could lead to more effective prevention and treatment strategies for cardiovascular diseases.

Further emphasizing the significance of oxidative stress in cardiovascular pathology, Strobel et al. (2011) explored the relationship between oxidative stress biomarkers, including

the products of NADPH oxidase activity such as oxidized LDL (OxLDL), and cardiovascular disease (CVD). Their findings indicate a complex relationship where biomarkers show potential in predicting CVD outcomes. However, they also point out the need for more precise methods to assess oxidative stress in vivo, indicating that current techniques might not fully capture the nuances of NADPH oxidase's contribution to cardiovascular health.

In line with these observations, Trpković et al. (2015) reviewed the role of OxLDL as a biomarker of CVD, a product directly linked to the activity of NADPH oxidases. Their research highlights OxLDL's association with all stages of atherosclerosis, from early atherogenesis to more advanced stages of cardiovascular diseases, including hypertension, coronary, and peripheral arterial disease. This association suggests that the oxidative stress mediated by NADPH oxidase not only contributes to the initial stages of cardiovascular disease but also plays a crucial role in its progression.

Kumar et al. (2010) delves into the protective potential of antioxidants against oxidative stress-induced cardiovascular diseases, highlighting the detrimental impact of NADPH oxidase. Their review suggests that a diet rich in natural antioxidants could mitigate the adverse effects of oxidative stress on the cardiovascular system, pointing towards dietary interventions as a potential strategy to counterbalance the oxidative stress generated by NADPH oxidase activity.

2.3 Genetically modified animal models in NADPH oxidase research

2.3.1 NOX

Genetically modified animal models have significantly contributed to advancing our understanding of the roles played by the Nox/Duox family of NADPH oxidases in health and disease. This includes physiological and pathological issues related to reactive oxygen species (ROS) derived from Nox/Duox (Rivera et al., 2010). Studies on *NOX4*-deficient animals have demonstrated the dual role of these enzymes in disease, showing that the absence of NOX-derived ROS can sometimes worsen disease symptoms (Rivera et al., 2010). Using knockout animals has been indispensable in the identification of specific roles and regulatory mechanisms of Nox/Duox proteins across different physiological and pathophysiological states (Rivera et al., 2010). For example, Holterman et al. (2014) reported that podocyte-specific NADPH oxidase 5 expression contributed substantially to NADPH oxidase-dependent

production of reactive oxygen species, which resulted in glomerular podocyte dysfunction. These findings highlight the significance of understanding the biological functions of NADPH oxidases in disease pathogenesis.

Continued exploration of Nox/Duox-deficient animal models is crucial for deepening our comprehension of the balance of ROS in maintaining health and developing targeted therapies for disease prevention and treatment (Rivera et al., 2010). By utilizing genetically modified animal models, researchers can further elucidate the specific gene expression changes induced by cholesterol rich diets in *NOX5* gene knockout rabbit models, shedding light on the interplay between oxidative stress and disease progression (Rizvi et al., 2012).

According to a study conducted by Sirokmant et al. (2016), the Nox/Duox family of enzymes plays a crucial role in controlling ROS production, as uncontrolled levels of ROS can lead to disease and cellular damage. In their study, they utilized knocked out mouse models to gain significant insights into the functions of the Nox/Duox enzymes (Sirokmant et al., 2016). The study further reported that the absence of Nox/Duox enzymes can alter the progression and development of diseases. For example, *NOX1* deficient mice exhibited changes in blood pressure regulation and inflammatory bowel disease, while *NOX3* knockout mice displayed balance disorders. *NOX4*, which is found in the lungs, heart, and kidneys, plays a protective role in stress conditions, and the absence of *NOX4* can exacerbate disease severity related to these organs (Sirokmany et al., 2016). This research highlights the therapeutic potential of targeting these enzymes.

2.4 *NOX5* KO rabbit

2.4.1. The experimental rabbit why is it a good model animal

Transgenic rabbits, especially those with specific gene knockouts like the *NOX5* gene knockout (KO) rabbit model, offer unique advantages as animal models for studying various diseases and conditions. The *NOX5* KO rabbit model is particularly valuable for research purposes due to several characteristics. The *NOX5* gene is known to participate in the generation of reactive oxygen species (ROS) (Rizvi et al., 2012). Researchers can knock out this gene in rabbits and assess how changing ROS levels affect various physiological processes such as the oxidant-antioxidant system affected by a cholesterol rich diet. This model allows for studying complex interplay between oxidative stress and development of diseases by

providing understanding at possible therapeutic targets. To add on that, rabbits are closer to humans phylogenetically than rodents which makes them more suitable for modeling certain human diseases (Yuan et al., 2019). Similarities in terms of genetics and physiology between humans and rabbits increase the translational importance of rabbit models' findings to clinical applications. Concerning the study on effects of cholesterol rich diet on oxidant-antioxidant system, *NOX5* KO rabbit model can offer valuable data that may better reflect human responses compared to other animal models. Also, genetic engineering techniques including CRISPR/Cas9-mediated gene editing have progressed such that it's possible to create accurate and reproducible gene knockout in rabbits (Zha et al., 2021). This level of precision helps in the development of consistent experimental models that guarantee reproducibility and reliability of research outcomes. Precise generation of targeted gene knockouts, such as the *NOX5* KO rabbit model, allows scientists to study specific gene functions and their involvement in disease processes more accurately.

According to Bosze et al., 2016, the rabbit is an excellent model for cardiac studies due to its physiological resemblance to humans. In the past, transgenic rabbits were created through pronuclear microinjection, which was a low-efficiency method with variable gene expression. But now with advanced methods like Sleeping Beauty Transposon System and TALENs (Targeted genome engineering tools) ZFNs (Zinc Finger Nucleases), CRISPR/Cas system has considerably increased accuracy and generation efficiency (Bosze et al., 2016).
CRISPR/Cas outshines other methods in generating multi-gene knockouts that are relevant for cardiovascular diseases. On the other hand, TALENs and ZFNs remain important for cases needing high specificity in genetic alterations (Bosze et al., 2016). These advances help researchers simulate human heart conditions in rabbits thus giving them deep understanding on heart diseases and helping them develop new treatments. Future improvements in gene editing accuracy and the use of bioinformatics are expected to further refine the development of rabbit models for cardiac studies, promising significant contributions to translational medicine (Bosze et al., 2016).

2.4.2 *NOX5* knockout rabbit

According to the study performed by Petheö et al., 2021, *NOX5* gene disruption in rabbits can worsen atherosclerosis by increasing plaque formation in thoracic aortas of *NOX5* knockout rabbits compared to wild-type rabbits. In this study, CRISPR/Cas9 technology was used to knock out the *NOX5* gene in New Zealand White rabbits, which allowed for the generation of *NOX5* knockout rabbits. The experiment showed that *NOX5* knockout resulted in more plaque formation in the aorta following an atherogenic diet implying that *NOX5* may be protective against atherosclerosis in young male rabbits (Petheö et al., 2021).

The research findings suggest that *NOX5* may play a protective role in atherosclerosis development in rabbits, contrary to previous studies in mice that did not show a pro-atherosclerotic effect of *NOX5* (Batty et al., 2022). This experiment was based on the gene family known as Nox/Duox consisting of NADPH oxidases involved in regulated reactive oxygen species production. Although the precise mechanisms by which *NOX5* protects against atherosclerosis are still not clear enough, these results give some valuable suggestions about how *NOX5* might be important for the proper functioning of vascular health (Batty et al., 2022; Petheö et al., 2021).

The study also highlights the importance of investigating *NOX5* in non-rodent models like rabbits, where the *NOX5* gene is present. This research contributes to expanding the understanding of the role of *NOX5* in vascular pathologies and suggests that *NOX5* may have context-dependent effects on atherosclerosis development, emphasizing the need for further mechanistic studies to clarify the precise impact of *NOX5* in cardiovascular diseases (Petheö et al., 2021; Batty et al., 2022).

2.5 Antioxidant system

The antioxidant defense mechanisms within endothelial cells involve a complex interplay of genes and processes that regulate redox homeostasis. Key genes involved in this system include NF-E2-related factor 2 (*NRF2*) and its downstream targets. *NRF2* acts as a transcriptional activator that regulates the expression of genes controlled by antioxidant response elements (ARE) during cellular response to oxidative stress (Satta et al., 2017). *KEAP1* binds to *NRF2* under normal situations leading to its degradation and low levels. However, oxidative stress results in *KEAP1* releasing *NRF2* from binding, becoming stable, accumulated and translocated into the nucleus where protective gene expression begins. In

order to scavenge ROS and RNS, these genes include phase II antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), peroxiredoxin (PRX) among others which play vital roles (Zhang et al., 2013).

The study performed by Panday et al., (2020) explains the *GPX glutathione* (GSH) are important in maintaining cellular redox balance (Raghunath et al., 2018). The depletion of GSH especially through the action of N2O3 is examined as an important mechanism influencing antioxidant levels in a switch-like manner (Raghunath et al., 2018). Additionally, the research underscores the cooperative function of *PRX* with GSH and *GPX* in detoxifying ROS and RNS, emphasizing the intricate network of antioxidant enzymes involved in combating oxidative stress (Raghunath et al., 2018). The study challenges existing notions by revealing that the activity of the GSH/GPX system is largely independent of physiological NADPH levels, suggesting a unique regulatory mechanism in endothelial cells (Raghunath et al., 2018). This finding shows that redox regulation is complex and underscores the need for understanding comprehensively the network of antioxidant defense mechanisms. To regulate their activity, various genes interact with one another in this antioxidant system, for example through the participation of *NRF2* interacting with *PRX, SOD,* and *GPX*. Understanding the regulatory mechanisms of these genes and their interactions is important for maintaining cellular redox homeostasis and combating oxidative stress which has implications for therapeutic interventions targeting vascular pathologies.

2.5.1 *NRF2* and *KEAP1* Gene

The *NRF2* gene also known as *NFE2L2* is considered an important regulator of the cellular response to oxidative stress. This gene encodes a transcription factor which is crucial in activating genes with antioxidant response elements (ARE) in their promoters. The *KEAP1* complex degrades *NRF2* in the cytoplasm under normal conditions. But during oxidative stress *NRF2* accumulates in the nucleus and binds to ARE elements to promote the cytoprotective gene expression and provide defense against oxidative damage (Hybertson & Gao, 2014). This study also indicates that regulation of the *NRF2* pathway protects cells against stress. Under normal conditions *NRF2* is bound to *KEAP1* which further leads to the breakdown of *NRF2* in the cell. When stressors such as phytochemicals or oxidants are present they cause the release of *NRF2* from *KEAP1*. Now free *NRF2* moves to the nucleus and turns on the transcription of protective genes that help the cell resist oxidative stress (Figure 4).

According to a study performed by Pajares et al., 2016, any alteration in the NFE2L2 gene can cause various disorders such as developmental delay, and hyperhomocysteinemia. Variations in NFE2L2 have been linked with lung squamous cell carcinoma and other cancers. This highlights the significance of NFE2L2 in cellular defense mechanisms and maintaining homeostasis (Qian et al., 2015). *KEAP1* is broadly expressed in various tissues, with implications in diseases such as Goiter and lung cancer, particularly in non-small-cell lung cancer (Binkley et al., 2020).

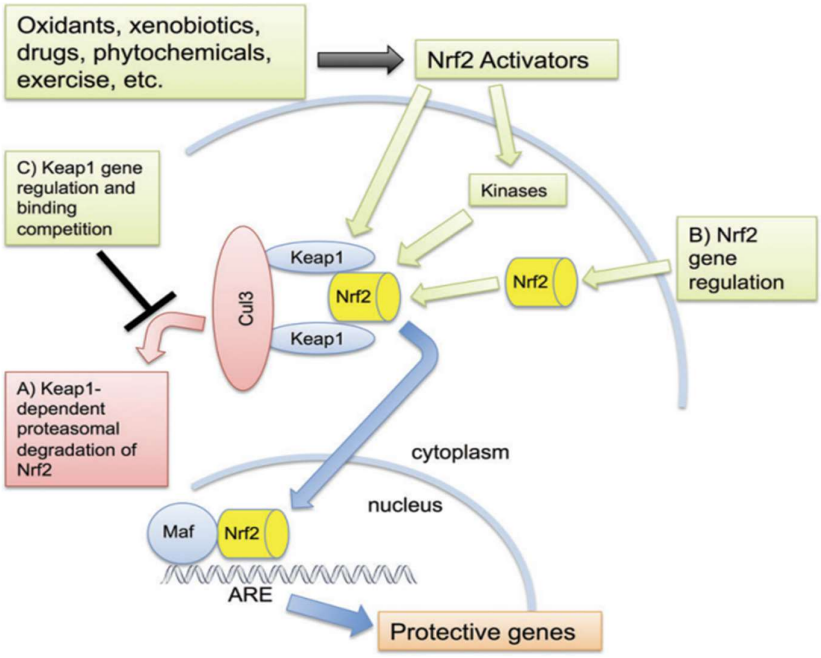

Figure 4: *NRF2* signaling pathway (Hybertson & Gao, 2014).

2.5.2 SOD1, SOD2, SOD3 Gene

SOD1, also known as Cu/Zn superoxide dismutase, is predominantly located in the cytoplasm. It is responsible for protecting cells from superoxide radicals by converting them into molecular oxygen and hydrogen peroxide, and by doing this it plays an important role in cellular defense in response to oxidative stress. Ju et al., 2020 in their study explains that mutations in *SOD1* are linked with neurological conditions and Amyotrophic Lateral Sclerosis 1 (ALS1), emphasizing its importance in cellular defense mechanisms. Further, studying this

could help in understanding the response of cytoplasmic oxidative stress in *NOX5* knockout versus wild-type animals, potentially illuminating the mechanisms underlying atherosclerosis development.

SOD2, also known as manganese superoxide dismutase, is important for detoxifying reactive oxygen species (ROS) within cells. *SOD2* gene is linked to various conditions that occur because of oxidative stress such as premature aging, cardiomyopathy, cancer, and sporadic motor neuron disease (Wang et al., 2018). This is related to heart health as cardiac conditions are influenced by oxidative stress and antioxidant activity of *SOD2* can potentially mitigate oxidative damage and affect the atherosclerotic lesions development (Ballinger et al., 2002). Understanding genetic variation in *SOD2* is very important for assessing susceptibility of individuals to oxidative stress related damage and cardiac conditions. (Ballinger et al., 2002).

SOD3, or extracellular superoxide dismutase is present in blood vessels and extracellular matrix. This gene is responsible for converting superoxide radicals into oxygen and hydrogen peroxide (less harmful species) and helps in protecting tissues from oxidative stress (Zhang et al., 2022). According to study performed by Zhang et al., 2022, if any variation occurs in *SOD3* gene it can increase chances of heart conditions. This study also explains that functions of *SOD3* in the extracellular matrix contribute to antioxidant defense mechanisms (Zhang et al., 2022). This gene prevents oxidative damage in the brain and lungs.

2.5.3 *CAT* Gene

The gene *CAT* which encodes catalase, is an important antioxidant enzyme that plays a key role in the defense of reactive oxygen species (ROS) to hydrogen peroxide by converting it into water and oxygen (Li et al., 2021). It is important in inhibiting oxidative damage to the cell and maintaining redox balance, at the same time (Li et al., 2021). It is thought that oxidative stress is involved in the development of chronic diseases such as Alzheimer's disease, asthma, diabetes, and cancers, along with rheumatoid arthritis. Variation in *CAT* gene can affect the catalase activity and results in conditions such as vitiligo and male infertility. Acatalasemia is the only disease that has been identified which is directly linked to mutations in this *CAT* gene (Liu et al., 2010; Sadia et al., 2021).

2.5.4 *GPX1* Gene

The *GPX* gene family produces enzymes called glutathione peroxidases, necessary in defending bodies from oxidative damage by reducing hydrogen peroxide and organic hydroperoxides into harmless substances. These enzymes are widespread across the body, with each type having its specific role and location. *GPX1*, the most abundant form, is found in the cytosol of nearly all mammalian tissues, playing a general role in cellular defense against oxidative damage (Rajput et al., 2021). *GPX4* is unique as it specifically targets lipid peroxides within biological membranes and lipoproteins, preventing the oxidative degradation of lipids that could lead to cell membrane disruption or the formation of atherosclerotic plaques. Study performed by (Lubos et al., 2011), showed that their activity relies on selenium, a necessary trace element that highlights the importance of a balanced diet for optimal enzyme function. Dysfunctions or mutations in these genes can lead to increased susceptibility to oxidative damage, emphasizing the importance of the *GPX* gene family in maintaining cellular health and preventing disease.

2.5.5 *PRDX* Gene

According to the National Center for Biotechnology Information, there are a number of genes which belong to the peroxiredoxin (PRDX) family and these include *PRDX1*, *PRDX2*, *PRDX3*, *PRDX4*, *PRDX5* and *PRDX6*. These mainly serve as a natural defense mechanism against oxidative stress by reducing hydrogen peroxide and alkyl hydroperoxides. *PRDX1* and *PRDX2* have been found in cytosol and play significant roles in CD8(+) T-cells antiviral activity and help protection against cancer cell injury (Rhee et al., 2005). *PRDX3*, *PRDX4*, and *PRDX5* also exhibit antioxidant properties involved in modulation of cell growth as well as differentiation. *PRDX3* is situated in mitochondria where its major role is maintaining mitochondrial integrity through diminishing mitochondrial peroxides to prevent aging and neurodegenerative diseases.

PRDX4 is located in extracellular space and endoplasmic reticulum and extracellular space (Rhee et al., 2005). It helps in secretion processes and protein folding and also in managing oxidative stress that is related to endoplasmic reticulum stress, with implications for diabetes and atherosclerosis. *PRDX5* is widely distributed in the body and is found in mitochondria, peroxisomes, cytosol, and the nucleus. It is involved in redox signaling and

protects the cell's nitro-oxidative stresses. *PRDX6* is present in lysosomes and cytosol and reduces H(2)O(2) and short chain organic, fatty acid, and phospholipid hydroperoxides. It is required for protection against oxidative injury and phospholipid turnover regulation. These peroxiredoxins are required in maintaining cellular homeostasis (Rhee et al., 2005).

2.6 Role of *NOX5* in brain function

The role of *NOX5* gene in brain function has been explored in recent studies, shedding light on its implications for vascular health, blood-brain barrier (BBB) integrity, and cognitive function. According to a study carried out by Cortés et al. 2021, it explains the impact of endothelial *NOX5* expression on BBB integrity and cognitive function in aging mice. Their findings indicated that *NOX5* expression led to increased oxidative stress, compromised BBB integrity, and significant memory impairments in aged mice. It has been observed that *NOX5* is important in reducing oxidative stress related to cognitive decline during and BBB disruption.

In response to this another study performed by Casas et al. 2019, explains that molecular mechanisms underlying ischemic stroke exacerbate post-reperfusion therapy by focusing on *NOX5's* contribution to BBB breakdown. This study examined *NOX5* as a main mediator related to oxidative stress following ischemic events, worsening brain damage by producing ROS and compromising BBB. This research highlights the potential of *NOX5* inhibition as a therapeutic strategy to limit stroke-induced damage by preserving BBB integrity. Casas et al., 2019, also highlighted the contribution of *NOX5* to BBB breakdown and increased brain damage post stroke by being a key source of ROS upon reoxygenation or calcium overload. The study highlighted that inhibiting *NOX5* could be a potential therapeutic strategy to reduce post-stroke damage and it also highlights the significance of preserving BBB integrity to improve stroke outcomes (Casas et al. 2019). This research also explained that *NOX5* is responsible for acute surge in ROS production following glucose and oxygen deprivation which indicates its function in early post-stroke ROS generation. Cases et al., 2019 performed studies on *NOX5* -KI mice and resulted in increased formation of ROS, worse neurological outcomes, and larger infarct volume when compared to wild type mice. This highlights the worsening effect of *NOX5* on brain damage. The study also explained that pharmacological inhibition of *NOX5* prevented reoxygenation-induced BBB permeability enhanced in the human BBB model which suggested that *NOX5* contributes to early BBB disruption post-

stroke. ML090 is the potential therapeutic inhibitor of *NOX5* that helps in preserving integrity of BBB and improve stroke outcomes (Casas et al. 2019).

Marqués et al., 2022 in their study explains the role of *NOX5* in facilitating endothelial dysfunction which is a precursor to cardiovascular diseases. Through inducing overexpression of *NOX5* in human brain microvascular endothelial cells they have examined enhanced production of ROS, altered migration, apoptosis, and metabolic changes. This finding connected the overexpression of *NOX5* to detrimental impacts on the viability of endothelial cells and suggested its contribution in vascular pathology and potential contribution to the development of cardiovascular diseases (Marqués et al., 2022). The destructive effect of *NOX5* on the brain was emphasized due to lack of exacerbated outcomes in models of heart ischemia and peripheral artery disease. This indicates the importance of *NOX5* in BBB disruption and stroke pathology. It explains the importance of targeting *NOX5* in the context of stroke to protect the BBB and reduce brain damage (Casas et al. 2019). Future studies will need to determine the optimal therapeutic window and administration method for *NOX5* inhibitors to maximize their benefits in stroke treatment.

2.7 High cholesterol affects the brain and its function

High cholesterol has been linked to affecting the brain and its function, particularly in the context of neurodegenerative disorders like Alzheimer's Disease (AD). In a study performed by (Zhao et al., 2016), indicates that high cholesterol levels have a direct impact on the metabolism of amyloid precursor protein (APP) leading to an increase in amyloid-beta (Aβ) production, which is a key factor in Alzheimer's Disease (AD) pathogenesis. This study also indicates that alterations in cholesterol levels influence cell membrane properties, especially in cholesterol-rich membrane microdomains known as lipid rafts, where key enzymes involved in Aβ production such as BACE1 and γ-secretases, are located (Zhao et al., 2016). The increase in membrane cholesterol enhances the binding of APP to lipid rafts and facilitates their breakdown into Aβ by BACE1 and γ-secretase through the amyloid metabolic pathway. Intracellular cholesterol levels play a crucial role in the activity of secretases involved in the APP metabolic pathway. Elevated intracellular cholesterol levels have been associated with increased activities of BACE1 and γ-secretase, leading to higher Aβ generation. High cholesterol levels not only elevate Aβ production but also promote the conversion of soluble Aβ monomers into aggregated and toxic structures which results in the development of AD.

Therefore, the study performed by (Zhao et al., 2016), concludes that hypercholesterolemia contributes significantly to the progression of AD by disturbing cholesterol homeostasis which leads to the formation of amyloid-beta (Aβ) plaques and tau protein hyperphosphorylation.

In conformity to (Zhao et al., 2016) study, another study performed by Jin et al. (2018) describes that Alzheimer's is characterized by the accumulation of β-amyloid (Aβ) plaques and neurofibrillary tangles, leading to synaptic loss and neurodegeneration, which manifest as memory impairment and cognitive decline. This study signifies the role of cholesterol in AD pathogenesis, where it influences the formation, aggregation, and neurotoxic effects of Aβ. In this research (Jin et al., 2018) used cholesterol-fed rabbits and advanced Magnetic Resonance Imaging (MRI) techniques and reported that a high-cholesterol diet led to decreased ratios of N-acetylaspartate to creatine (NAA/Cr) and glutamate to creatine (Glu/Cr), suggesting neuronal loss or dysfunction and alterations in neurotransmitter systems (Jin et al., 2018). These findings parallel metabolic changes observed in AD, reinforcing the link between high cholesterol and AD pathology. Structural MRI showed brain atrophy in cholesterol-fed rabbits, including reduced volumes in the cortex and hippocampus, which are indicative of the structural changes seen in AD (Jin et al., 2018).

Another study performed by (Dias et al., 2014), explains that hypercholesterolemia can induce oxidative stress at the blood-brain barrier. This oxidative stress can result in irreversible damage to proteins, lipids, and DNA, potentially impacting brain function. This research demonstrated that oxidative stress from high cholesterol levels leads to the production of radical oxidized lipids, which are more prevalent in individuals with Alzheimer's disease (Dias et al., 2014). In support of this study, (Norton et al., 2013), describes that oxidative stress can have implications for brain health, potentially affecting cognitive function. Studies have identified elevated plasma cholesterol levels in mid-life as an independent risk factor for Alzheimer's disease (Norton et al., 2013).

2.8 Choosing of the ideal housekeeping gene: HPRT vs YWHAZ

During brain studies, the selection of an appropriate housekeeping gene for the normalization of gene expression is important for reliable and accurate results. In a study performed by (Lie et al., 2015) indicates that rat dorsal root ganglia (DRG) neurons examined hypoxanthine phosphoribosyl transferase (*HPRT*) as the most stable reference gene for the analysis of quantitative real-time reverse transcription polymerase chain reaction (qPCR)

analysis. The suitability of *HPRT* as a housekeeping gene for brain studies can be argued from its stable expression under different treatments or across various developmental stages. This study further indicates that *HPRT* is known for its involvement in fundamental cellular functions, showing negligible changes in cycle threshold (Ct) values among different developmental stages and treatments in DRG neurons (Lie et al., 2015). Researchers have confirmed the stability of *HPRT* through various evaluation methods, including NormFinder and geNorm analyses, and concluded *HPRT* as one of the most stable reference genes. In this study (Lie et al., 2015), used 12 reference genes and checked how they behave under distinct conditions. Further, the finding of this study indicates that among 12 reference genes, including *HPRT*, β-actin , 18S ribosomal *RNA*, mitochondrial ribosomal protein L10 (Mrpl10), ubiquitin C (Ubc), etc. 18S ribosomal *RNA* (Rn18s) and β-actin (Actb), showed significant variations in their expression levels, while ubiquitin C (Ubc) and mitochondrial ribosomal protein L10 (Mrpl10), demonstrated minor changes. Thus, this suggests *HPRT* as a stable reference gene enhances the accuracy and validity of gene expression studies in the context of brain research. Based on the research article above, We initially selected *HPRT* as our reference gene. However, due to issues with sample quality and observed discrepancies, we decided to conduct a parallel comparison between *HPRT* and *YWHAZ*. This analysis showed that *YWHAZ* exhibited greater stability among our samples, leading us to choose it as the more suitable reference gene for our study. In another study performed by Kim et al., 2023, explains that *YWHAZ* were more stable reference genes among 14 assessed housekeeping genes in their study. The stability of reference genes was assessed using tools like RefFinder, that integrate results from multiple algorithms (Delta-Ct, geNorm, NormFinder, and BestKeeper) to rank the stability of the gene. This research positions *YWHAZ* as a promising reference gene for accurate gene expression analysis.

2.9 Summary of Existing Literature

The summary of existing literature on the investigation of the oxidant-antioxidant system in NADPH5 as a response to a cholesterol-rich diet is given in Table 1.

Table 1: Summary of Existing Literature

Reference	Summary
Chen et al., 2016	The result of this study showed the cloning and functional analysis of rabbit *NOX5,* highlighting the suitability of rabbits as models for studying the role of *NOX5* in cardiovascular diseases, while also focusing the need for further research on the significance of *NOX5* in various diseases. The phylogenetic analysis sheds light on the genetic relationship between rabbit *NOX5* and *NOX5* in other species.
Touyz et al, 2019	The study focuses on the significance of NADPH oxidases in generating ROS and their implications in cardiovascular diseases, emphasizing the need for further research to understand the specific roles of different Nox isoforms, especially *NOX5,* in human health and disease.
Rivera et al., 2010	The finding of this research explains the roles of NADPH oxidases in vascular disease, emphasizing the contributions of *NOX1* and *NOX2* isoforms to elevated ROS production while noting the lack of information on the effects of genetic manipulation of *NOX4* or *NOX5*.
Jha et al., 2017	The emerging role of NADPH oxidase *NOX5* in vascular disease, highlighting its absence in rodents and its up-regulation in humans, critically analyzes its pathophysiological role in disease. *NOX5* is highly up-regulated in humans in disease. This study discusses the increasingly recognized function of NADPH oxidase *NOX5* in vascular diseases. It emphasizes that *NOX5* is not present in rodents but is significantly upregulated in humans. It also explains that *NOX5* is highly up-regulated in humans in disease.
Petheő et al., 2021	The performed study focuses on the role of *NOX5.* Unlike other NOX enzymes, *NOX5* is less understood and was primarily found in testis and lymphoid tissues. Due to the absence of *NOX5* in rodents, researchers used CRISPR/Cas9 to knock out the *NOX5* gene in New Zealand White rabbits, revealing a potential protective role of *NOX5* against atherosclerosis. Thus, this study evidenced that *NOX5* could be involved in vascular activity, highlighting the need for further studies to understand the functions and regulatory mechanism of *NOX5* gene.

3. MATERIALS AND METHODS

3.1 Animal Model and Diet Regimen

For this study young male New Zealand white rabbits were used (Figure 5), which were divided into two groups: *NOX5*-deficient (*NOX5*-/-) and wild-type (*NOX5*+/+), with all subjects being 20 weeks old at the onset. The *NOX5*-/- rabbits were generated using the methodology described by Petheő GL. et al., 2021. The animals were housed individually, ensuring they had constant access to food and water. The environment was controlled with a 12-hour light-dark cycle (06:00 – 18:00 h) and maintained at a steady temperature of 19°C.

Figure 5: Young New Zealand Rabbit

To induce atherosclerosis, the rabbits were subjected to a dietary regime starting with a 4-day period of mixed feeding (50% standard diet and 50% cholesterol rich diet) followed by 8 weeks on a cholesterol rich diet containing 0.5% w/w cholesterol. This dietary approach was chosen based on previous findings indicating that male rabbits exhibit less variability in cholesterol levels and are less impacted by hormonal changes than females, making them more suitable for consistent experimental outcomes. Before and after the cholesterol diet, researchers measured cholesterol and triglycerides levels and after termination of the animals checked for aortic plaque formation by looking at the rabbits arteries. Following the diet, both *NOX5*-/- and WT expressed increased levels of plasma cholesterol, however, rabbits without *NOX5* gene had

less severe atherosclerosis. This suggests a protective role of the absence of *NOX5* against atherosclerosis.

3.2 Sample Collection for the experiment

Upon completion of the dietary regimen, the rabbits underwent euthanasia to allow for the collection of tissue samples. This euthanasia was carried out in a humane manner, utilizing an intravenous injection of pentobarbital, dosed at 0.25ml of Euthanimal (Alfasan Int. B.V.) per kilogram of the rabbit's body weight. Subsequent to euthanasia, tissue samples (liver, kidney, brain, heart, spleen) have been cut out (and were immediately submerged in the RNAzolRT solution to ensure the integrity of *RNA* within the samples. The brain tissue used in my experiment was isolated from the right prefrontal cortex of the brain. After immersion in RNAzolRT, the samples were subjected to flash freezing in liquid nitrogen (-70°C) until *RNA* isolation could be conducted. Freezing helps to halt biological processes and prevents *RNA* decay or sample degradation over time. This methodological approach ensures that the collected samples are of the highest quality, appropriate for further examination.

Table 2: Sample Type and Treatment Group

Sample Number	Sample Type	Treatment Group
1	*NOX5*	Cholesterol Fed
2	*NOX5*	Cholesterol Fed
3	*NOX5*	Cholesterol Fed
4	*NOX5*	Cholesterol non-Fed
5	*NOX5*	Cholesterol non-Fed
6	*NOX5*	Cholesterol non-Fed
7	Wild Type	Cholesterol non-Fed
8	Wild Type	Cholesterol non-Fed
9	Wild Type	Cholesterol non-Fed
10	Wild Type	Cholesterol Fed
11	Wild Type	Cholesterol Fed
12	Wild Type	Cholesterol Fed

Based on table 2 provided, our experiment was structured around four distinct groups of brain tissue samples:

1. *NOX5* gene knockout rabbit fed with a cholesterol-rich diet.
2. *NOX5* gene knockout rabbit fed with a standard diet without added cholesterol.
3. Wild type rabbit fed with a cholesterol-rich diet.
4. Wild type rabbit fed with a standard diet without added cholesterol.

This grouping allowed us to systematically explore the effects of *NOX5* gene knockout and dietary cholesterol on our experimental outcomes.

3.3 *RNA* Isolation

Two different *RNA* isolation protocols, using RNAzol and TRIzol reagents, were compared to perfect the procedure of *RNA* isolation, to be conducted in the following experiment during gene expression analysis. Keeping that into consideration, we tried to optimize the best method in terms of quantity and quality of *RNA*, which are important key points for good downstream application.

RNA Isolation Methods Using RNAzol and TRIzol Methods

RNA was isolated from tissue samples using two different methods employing RNAzol and TRIzol reagents. The protocols followed are detailed below:

RNAzol Protocol:

1. Tissue samples were placed into new 1,5 ml Eppendorf tubes, with each tube receiving half of the sample, weighing about 100 mg.
2. 1 mL of RNAzol was added to each tube, followed by homogenization with zirconium beads using Mini Beadbeater Homogenizer (Figure 6 a).
3. After homogenization, 400 µL of RNA-free water was added to each sample.
4. Samples were centrifuged for 15 minutes at 4°C at 13000 rpm.
5. The supernatant was transferred to a new Eppendorf tube.
6. Equal volumes of isopropanol were added to the supernatant, followed by incubation for 10 minutes at room temperature.

7. Samples were centrifuged again for 10 minutes at 4°C at 12000 rpm for *RNA* pellet collection.
8. The supernatant was removed with the help of a pipette tip.
9. 600 µL of 75% ethanol was added in the *RNA* pellet and centrifugation was done for 3 minutes at room temperature at 6000 rpm.
10. The sample was washed twice more using a pipette tip and then dried.
11. The *RNA* pellet was resuspended in RNA-free water.
12. Samples were vortexed for 2-3 minutes.
13. The concentration of the *RNA* was measured using a Nanodrop spectrophotometer (Figure 6 b).

TRIzol Protocol:

1. Tissue samples ranging from 50-100 mg were placed in 1,5 ml Eppendorf tubes.
2. In each tube 1 mL of TRIzol reagent was added, followed by homogenization with 1.0 mm zirconium beads (Gentaur) for 5 -7 minutes using Mini Beadbeater homogenizer.
3. The homogenized samples were incubated for 5 minutes at room temperature.
4. 0.2 mL of chloroform per 1 mL of TRIzol was added, followed by shaking.
5. Samples were incubated for 2-3 minutes at 4 °C and then centrifuged at 12000 rpm for 15 minutes at 4°C for phase separation.
6. The aqueous phase was transferred to a new Eppendorf tube.
7. *RNA* was precipitated from the aqueous phase by adding 0.5 mL of isopropanol per 1 mL of TRIzol used initially.
8. Following incubation for 10 minutes at 4 °C, the samples were centrifuged to pellet the *RNA* at 12000 rpm for 10 minutes at 4°C. (at this point we saw the pellet).
9. Supernatant was discarded and the pellet was resuspended in 1 mL of 75% ethanol per 1 mL of TRIzol.
10. The samples were vortexed and then centrifuged for 5 minutes at 7500 rpm at 4°C.
11. Supernatant was discarded and the *RNA* pellet was vacuum dried for 5-10 minutes.
12. Resuspend pellets in 30 microliters of RNase free water and incubate in a water bath for 10 minutes at 55-60 °C.
13. Both protocols included a final step of quantification and purity assessment of the *RNA* using a Nanodrop spectrophotometer.

Figure 6 (a): Mini Beadbeater Homogenizer, (b) Nanodrop Spectrophotometry

3.4 Gene Expression Analyses - qPCR

For gene expression analyses, 400 ng of total *RNA* was converted to *cDNA* using the VitaScript™ FirstStrand *cDNA* Synthesis Kit, following the manufacturer's protocol. Specific PCR primers were designed for the rabbit genes of interest using Primer3 software, ensuring amplification of the target gene segments.

qPCR Reaction

For the quantitative PCR (qPCR) assay, KiCqStart® SYBR® Green qPCR ReadyMix KCQS02 (Sigma) kit was utilized (Figure 8). First, the master mix was prepared by combining 5 µL of SYBR Green PCR Master Mix, 1.3 µL of RNase-free water, 1.2 µL of primers (0.6 µL each for forward and reverse), and 2.5 µL of *cDNA* (Table 3). Then distributed 7.5 µL of this mixture into each well of a PCR plate. Following the addition of 2.5 µL of the sample to each well. The plate was then centrifuged for 1 minute at 12,000 rpm and 4 °C to ensure optimal mixture and distribution of the contents.

Table 3: qPCR Reaction Mix for 10 µL Volume

qPCR Mix	1x
2X Mix	5 µL
RNase free water	1,3 µL
Primer forward	0,6 µL
Primer reverse	0,6 µL
cDNA (diluted)	2,5 µL
Total volume	10 µL

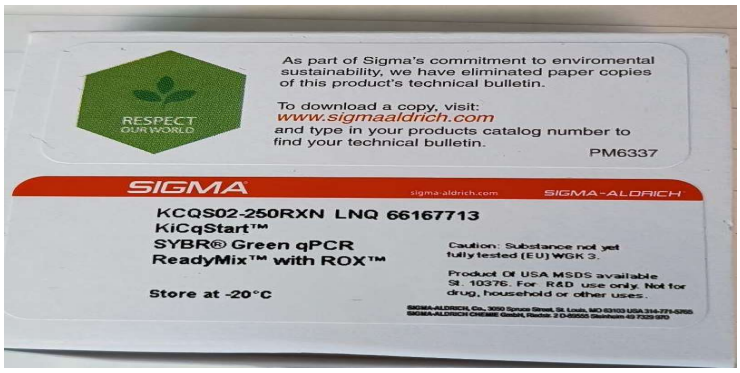

Figure 7: KiCqStart® SYBR® Green qPCR ReadyMix KCQS02 (Sigma) kit

Protocol for qPCR involves three steps:

1. Preincubation: In this step, samples were denatured at 95°C for 10 minutes to activate the Taq DNA polymerase to ensure complete denaturation of the DNA template.

2. Three Step Amplification

A. Denaturation: Each cycle started with a denaturation step at 95°C for 15 seconds.
B. Annealing: Next, the temperature was reduced to 60°C for 20 seconds to enable the primers to specifically bind to the target DNA sequence.
C. Extension: This step occurred at 72°C for 20 seconds, where the DNA polymerase synthesized new DNA strands.

3. High Resolution Melting (HRM): After amplification, HRM analysis was conducted to confirm the specificity of the PCR results. Melting curves were generated by gradually raising the temperature from 60°C to 95°C at a rate of 0.1°C or 0.4 °C per second, continuously monitoring the decrease in fluorescence.

4. Cooling: The reactions were then cooled to 40°C for 5 minutes to stabilize the amplified DNA and maintain the reaction mixture for subsequent analysis.
The temperature program of qPCR is described in Table 4.

Table 4: Temperature Program of the qPCR

Step	Temperature	Time	Number of Cycles
Preincubation	95°C	10 min	1
Denaturation	95°C	15 s	45
Annealing	60°C	20 s	
Extension	72°C	20 s	
High Resolution Melting	60°C to 95°C	0.1 or 0.4 ◦C/s	1
Cooling	40 °C	5 min	1

3.5 Primer Design and Sequences

Real-Time PCR (qPCR) is generally used to study gene expression in the molecular biology field, a very sensitive and accurate method of quantification. In each successful qPCR experiment, therefore, the insertion of primers at the core is necessary. Short, single-stranded DNA sequences capable of annealing to DNA and able to initiate DNA synthesis have been designed as primers.

An adequate selection of primers for genes has been made, those involved in the oxidative stress pathways and antioxidant defense system, giving good reflection of the thematic core in our study. Evidence had been previously presented on the rationale for choosing those genes, which were established to play crucial roles in the modulation of oxidative stress responses that account for part of the pathological scenery of cardiovascular diseases. The designing of the primer was done with the free online Primer3 software that uses stringent criteria so that one gets the maximum binding efficiency and at the same time, there

is a minimum formation of secondary primer-dimers or secondary structures. The primers for qPCR are described in Table 5.

Table 5: Primers for qPCR

Gene	Name	Forward	Reverse
NOX5	NADPH oxidase 5	ACTCGCGAACCGAAACTCTC	TCAGGCCAGGAACAGTTGTG
PRDX	Peroxiredoxin	AGGACATCGGGTTTCTGCG	GTGTTGATCCAAGCCAGGTG
CAT	Catalase	ACTCGCGAACCGAAACTCTC	AGGACATCGGGTTTCTGCG
HPRT	Hypoxanthine Phosphoribosyl transferase	ACGTCGAGGACTTGGAAAGGGTGTT	GGCCTCCCATCTCCTTCATCACATC
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta	GGTCTGGCCCTTAACTTCTCTGTGTTCTA	GCGTGCTGTCTTTGTATGATTCTTCACTT
SOD3	Superoxide dismutase 3	GAGTCAGCGGCTTGGTAGTC	CCCTCCAAGTCGAAGAAGGC

3.6 Analysis

In the study, expression fold change was the primary measure used to describe the change in levels of differential gene expression between groups of rabbits with different genetic background (*NOX5*-deficient vs. wild-type) exposed to different dietary regimens (Cholesterol fed and unfed). The fold change provides an intuitive way to easily understand increases in gene expression changes induced by both genetic background and dietary influence.

The first step was to normalize the gene expression data for variability due to differences in quantity and quality of the *RNA* samples from the samples. This was carried out using the expression levels of a housekeeping gene, *YWHAZ*, to make a reference.
In our study, we initially selected *HPRT* as the reference gene. However, due to instability of *HPRT* across the samples and inconsistencies in our preliminary data we conducted a parallel comparison between *HPRT* and *YWHAZ*. Our analysis demonstrated that *YWHAZ* showed superior stability across our samples, prompting us to select it as the reference gene for our study. This indicated that normalization brought the level of change in gene expression to depend on experimental conditions, not on the technical noise.

It was calculated as the ratio of normalized gene expression values in treatment groups (*NOX5* deficient, with both standard and cholesterol diet) to the respective values in the wild type control group (standard diet). A value of fold change greater than 1 connotes the upregulation of gene expression of the experiment condition, while the value falling below 1 connotes the downregulation of the gene with respect to the experiment condition. Fold change data were interpreted to give a summary of the likely effect of *NOX5* deficiency in conjunction with dietary cholesterol on the expression of genes involved in oxidative stress and those required for the antioxidant defense. Although fold change analysis is descriptive, it should still account for variability and importance in the change observed.
Results from the fold change analysis were visually represented through bar graphs.

3.7 Ethical Consideration

The research protocol was designed to adhere to the highest standards of animal welfare, in compliance with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes. Ethical approval was granted by the Animal Welfare and Research Ethics Committee affiliated with the Agricultural Biotechnology Institute, the National Agricultural Research and Innovation Centre, and the Hungarian University of Agriculture and Life Sciences Institute of Genetics and Biotechnology. All procedures were performed under the ethical permissions ÁK-ENG (PE/EA/58-2-2018) and PE/EA/00741-7/2022, issued by Pest County's governmental office, with a strong commitment to minimizing animal discomfort and stress.

4. RESULTS

4.1 Comparison of *RNA* Isolation Methods

This comparison aimed to evaluate the efficacy and purity of *RNA* isolation using two widely recognized reagents: RNAzol and TRIzol. Our analysis was twofold, focusing on the efficiency of *RNA* isolation and the integrity of the isolated *RNA*, which are critical for accurate gene expression studies. The data for concentration and contamination for both of the methods is given in Table 6.

RNA Isolation Efficiency

Quantitative assessments were performed using Nanodrop spectrophotometry to measure absorbance ratios (A260/A230) which is an indicator of organic contamination (Figure 8). Our findings showed that *RNA* samples isolated with the RNAzol protocol exhibited higher levels of phenol contamination than those obtained using the TRIzol method. This observation suggests that the TRIzol method may offer superior purity, making it more suitable for applications requiring high-quality *RNA*.

RNA Isolation Using RNAzol

Wild-Type rabbits without cholesterol fed varied significantly, in this sample 1 has a low concentration of 35.5 ng/µl and sample 6 a high concentration of 732.2 ng/µl. The purity ratios indicated by the A260/A280 values were mostly below the optimal range of 2.0, suggesting potential protein contamination as well. The A260/A230 values were generally low across all samples, with several values below the optimal range. This indicates contamination by organic compounds or chemicals used during the *RNA* extraction process.

RNA Isolation Using TRIzol

The concentration of *RNA* in *NOX5*-CH samples was relatively lower than the WT-0 samples, with concentrations ranging from 9.9 ng/µl to 123.1 ng/µl. A260/A280 ratios for the TRIzol-extracted samples were more variable, with some below the optimal range, suggesting

that some degree of protein contamination is present. A260/A230 ratios for the TRIzol-extracted samples were generally closer to the acceptable range, indicating less contamination compared to the RNAzol-extracted samples, although still presenting some degree of impurity.

Table 6: Comparison of *RNA* Isolation Methods Using RNAzol and TRIzol Methods

RNAzol			
Sample	ng/µL	A260/A280	A260/A230
1 WT - 0	35.5	1.92	0.05
2WT - 0	591.2	1.71	1.01
3 WT - 0	186.1	1.73	0.4
4 WT - CH	80.2	1.85	0.08
5 WT - CH	235	1.81	0.45
6 WT - CH	732.2	1.74	1.29
7 *NOX5* CH	18	1.33	0.86
8 *NOX5* CH	8.6	1.49	0.46
9 *NOX5* CH	114	1.56	0.55
10 *NOX5* -0	48.3	1.45	0.57
11 *NOX5* - 0	8.5	1.71	0.15
12 *NOX5* - 0	8.6	1.58	0.88

TRIzol			
Sample	ng/µL	A260/A280	A260/A230
NOX5 -CH	22	1.52	0.98
NOX5 -CH	9.9	1.45	0.47
NOX5 -CH	123.1	1.55	0.3
NOX5 -0	41.2	1.42	0.4
NOX5 -0	13.4	1.54	0.2
NOX5 -0	12.5	1.45	0.79
WT - 0	28.1	1.61	0.16
WT - 0	32.5	1.57	0.13
WT - 0	41.1	1.64	0.1
WT - CH	26.6	1.65	0.1
WT - CH	20	1.56	0.14
WT - CH	10.6	1.56	0.16

Comparison Between RNAzol and TRIzol

Comparing the two methods, the TRIzol method generally resulted in better purity based on the A260/A230 ratios but demonstrated a wide range of *RNA* concentrations. Conversely, the RNAzol method, while yielding higher *RNA* concentrations for certain samples, showed significant contamination issues that could potentially interfere with downstream applications.

The comparative analysis of the two methods of *RNA* isolation, RNAzol, and TRIzol, has shown a very important finding: the extraction method needs further optimization in order to be able to improve both the quality and yield of *RNA*. Therefore, several methods can be employed to assess *RNA* quality effectively. The Bioanalyzer, from Agilent, is a highly recommended tool for this purpose. It provides a detailed analysis of *RNA* integrity by generating an *RNA* integrity number (RIN), which quantifies the degradation level of *RNA* samples. This method offers a rapid and reliable means to evaluate *RNA* quality without the need for extensive sample preparation. Additionally, gel electrophoresis for *RNA* can serve as a more accessible and precise method to visually assess *RNA* integrity. Observing the distinct ribosomal *RNA* bands can give an indication of sample degradation and purity. These methods combined for checking *RNA* quality are key for ensuring that *RNA* samples meet the necessary standards for high-quality genetic analysis which leads to more reliable and accurate research outcomes.

Isolation of *RNA* from fatty tissues such as the brain poses specific challenges due to high lipid content and low cell numbers in these tissues which can interfere with purity and efficiency of *RNA* extraction. Studies show that fatty tissues contain a dense matrix and have high proportions of lipids that can encapsulate nucleic acids, which make them difficult to access and possibly result in decreased *RNA* quality and yields (Zhang et al., 2023). Efficient homogenization is important as traditional mechanical methods may not completely break down the dense, lipid-rich matrix. *RNA* in these tissues is degraded by enzymes. Therefore, *RNA* integrity must be maintained through effective inactivation. Considering these complexities, it is advised to use a standardized approach designed for fatty tissues is recommended for more reliable results (Zhang et al., 2023).

To address these complexities, a detailed protocol based on TRIzol reagent for isolation *RNA* from fatty tissues has been designed by (Zhang et al., 2023). This protocol involves the use of specialized lysis buffers that helps in solubilizing membranes and also disrupt lipid-*RNA* interactions, phase separation techniques to minimize lipid contamination, and modifies alcohol precipitation steps to enhance *RNA* purity. For future research, it is advisable to implement this standardized method to enhance *RNA* extraction efficiency from fatty tissues like the brain.

4.2 Testing of Primers

In quantitative polymerase chain reaction (qPCR) melting peak refers to a critical phase of the process where the amplification products are analyzed for their melting characteristics. After the amplification step a gradual increase in temperature occurs which causes the double-stranded DNA to denature or melt. This denaturation leads to the separation of the DNA strands and as the temperature rises the DNA strands separate at specific temperatures unique to their sequences.

The melting peak analysis in qPCR involves monitoring the fluorescence emitted by the DNA-binding dye (such as SYBR Green in our case) as the temperature increases. Each amplicon or target DNA sequence will have a characteristic melting temperature (Tm), which is the temperature at which 50% of the DNA strands are denatured. The melting peak is represented graphically as a peak on a plot of fluorescence intensity versus temperature.

To ensure the specificity and efficiency of our qPCR assays we conducted an initial screening of all primer pairs designed for the amplification of target genes including *SOD3, CAT, PRDX,* and *NOX5*. The primer pairs were evaluated based on their melting curves which is an essential step to identify non-specific amplification or primer-dimer formation which can affect the accuracy of gene expression analysis. Each primer pair was tested under identical conditions to obtain comparable results.

The melting curve analysis for each primer pair was performed following qPCR amplification where a single sharp peak represents specific amplification of the target gene whereas multiple peaks or a broad melting peak indicate non-specific products or primer-dimer formations.

Figures 8-11 of melting peak curves for *SOD3, CAT,* and *PRDX* Primer Pairs: The melting curve analysis for these genes showed a single distinct peak, indicative of specific amplification without significant primer-dimer formation or non-specific amplification. This suggests that the primer pairs designed for *SOD3, CAT,* and *PRDX*, are suitable for further experiments involving gene expression analysis.

NOX5 Primer Pairs: In contrast the primer pairs for *NOX5* exhibited melting curves with multiple peaks or unusually broad peaks (Figure 12), suggesting non-specific amplification and the presence of primer-dimers. Given the importance of primer specificity for accurate qPCR results, these primer pairs were deemed unsuitable for accurate gene expression analysis. The redesign and testing of new primer sets are scheduled for completion in our current project queue.

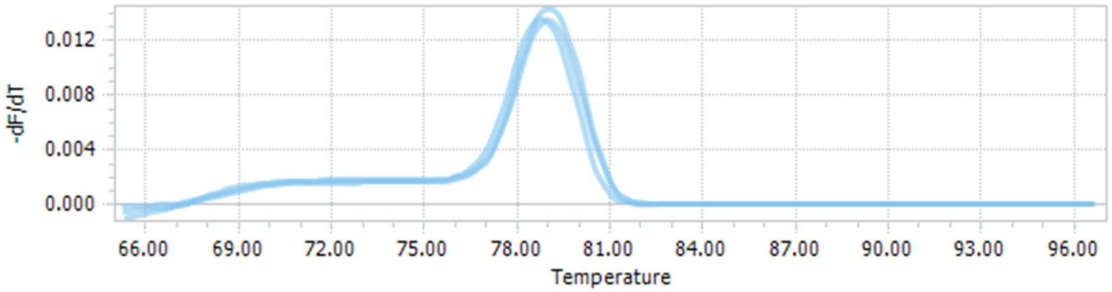

Figure 8: Melting peaks curve for *PRDX* gene

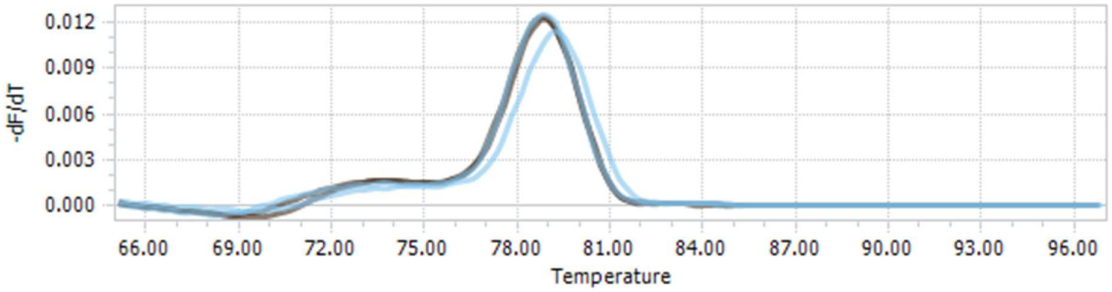

Figure 9: Melting peaks curve for *YWHAZ* gene

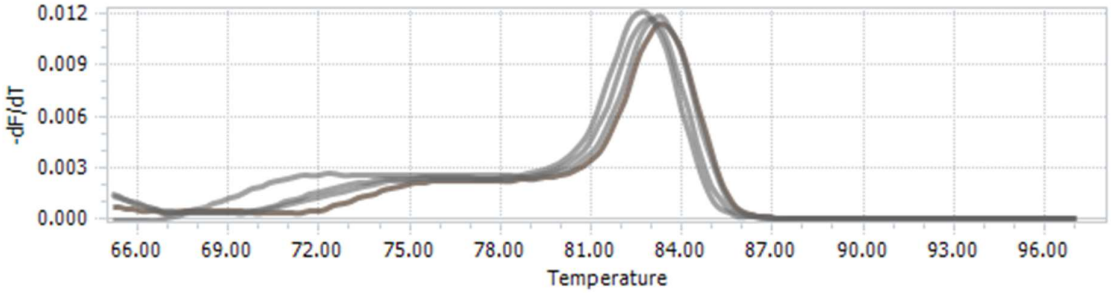

Figure 10: Melting peaks curve for *SOD3* gene

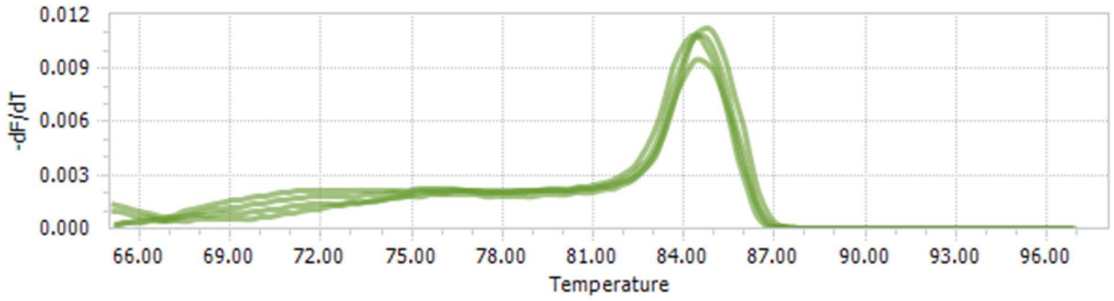

Figure 11: Melting peaks curve for CAT gene

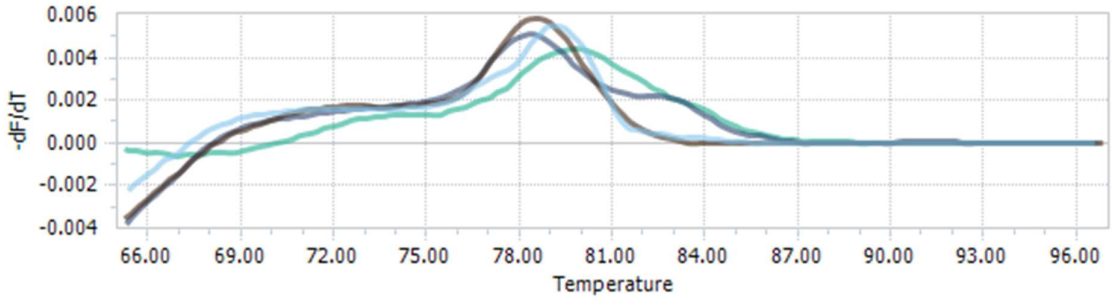

Figure 12: Melting peaks curve for *NOX5* gene

The melting curve analysis serves as a critical quality control step in the validation of primer pairs for qPCR. Specific amplification, as evidenced by a single sharp melting peak, is crucial for accurate quantification of gene expression levels. The identification of non-specific amplification or primer-dimer formation in the primer pairs for *NOX5* underscores the need for new primer design and/or optimization of the PCR reaction conditions for these targets. Non-specific amplification can lead to erroneous gene expression data, potentially skewing the interpretation of the biological significance of the results.

4.3 Relative expression for each gene

Our study aimed to elucidate the relative expression of key genes (*CAT, SOD3, PRDX, NOX5*) in *NOX5*-deficient (*NOX5*-/-) and wild-type (*NOX5*+/+) rabbits, both subjected to standard and cholesterol-rich diets. We employed *YWHAZ* as a housekeeping gene for

normalization. The following findings were derived from our quantitative Real-Time PCR (qPCR) analysis.

SOD3 (Superoxide Dismutase 3) **Gene Expression**

Table 7: *SOD3* Gene Expression

SOD3	DD-Ct (mean)
Wild Type - cholesterol non fed	1
Wild Type-cholesterol fed	0.094
NOX5 knockout -cholesterol non fed	5.259
NOX5 knockout -cholesterol fed	0.081

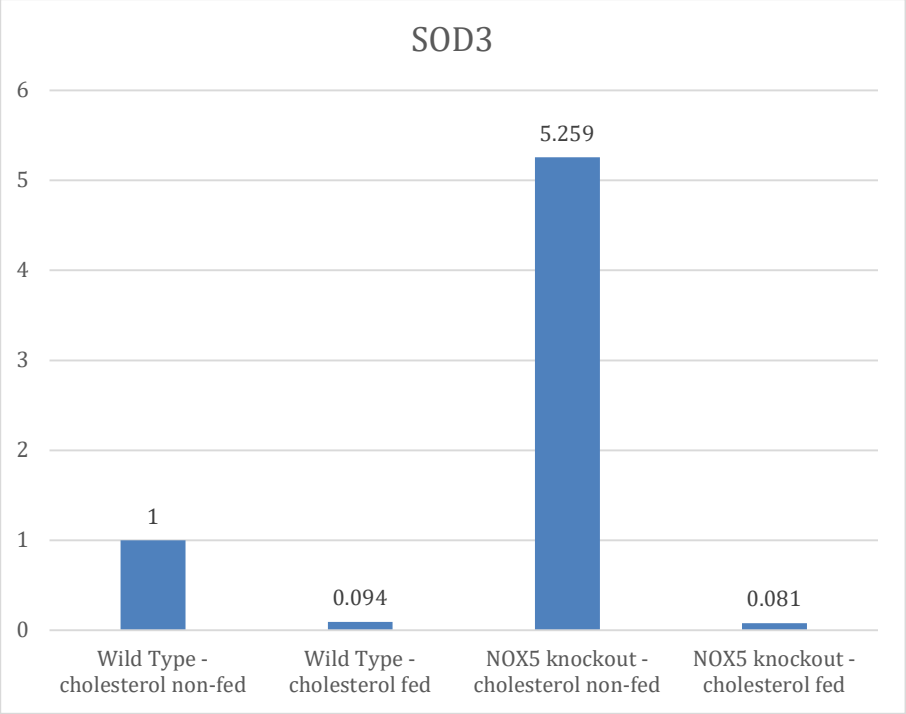

Figure 13: Bar graph for relative gene expression of *SOD3*

The relative gene expression of *SOD3* is described in Table 7 and Figure 13 which shows that wild-type rabbits on a standard diet indicated baseline expression levels, serving as the control group. Wild-type rabbits on cholesterol rich diets demonstrated a notable decrease in *SOD3* expression (Relative Expression: 0.094), suggesting that high cholesterol may suppress *NOX5* expression. *NOX5* -/- rabbits on standard diet exhibited elevated *NOX5* expression (Relative Expression: 5.259), it might indicate a response to the absence of *NOX5*.

However, several external and internal factors might lead to the activation of the antioxidant system, for example an ongoing infection that was not recognized, so these results are only indicative and needs to be reevaluated with larger experimental groups. *NOX5* -/- rabbits on cholesterol rich diet presented similar suppression in *NOX5* expression as wild-type animals under high cholesterol (Relative Expression: 0.081). These results suggest that while the absence of *NOX5* might trigger an increase in *NOX5* expression under standard dietary conditions, a cholesterol rich diet negates this effect, underlining the complex interplay between *NOX5* activity, diet, and antioxidant gene expression. Antioxidant response can decrease as a result of prolonged exposure to a cholesterol rich diet over the long term. This phenomenon primarily occurs due to several processes that also interact with each other. A diet high in cholesterol can increase the levels of LDL cholesterol and its continuous production can lead to persistent oxidative stress. As a result, the body uses more antioxidants to neutralize free radicals and this increased demand can deplete the body's reserves of endogenous antioxidants such as superoxide dismutase, catalase and glutathione. Also, the oxidative stress might disrupt normal cellular functions including those involved in the production of antioxidants. So, on the long run while antioxidants initially respond to counterbalance the oxidative stress caused by high cholesterol, their efficiency and levels can decline over time if the stress is overwhelming, leading to decreased antioxidant capacity and increased vulnerability to oxidative damage.

Superoxide Dismutase 3 (SOD3) is important for defenses in the body against oxidative stress as it helps in catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide. This process is vital for protecting cellular components from oxidative damage. The data obtained from our study on wild-type and *NOX5* -/- rabbits under different dietary conditions provide insights into the interplay between diet, genetic factors, and antioxidant defense mechanisms.

CAT (Catalase) **Gene Expression**

Table 8: *CAT* Gene Expression

CAT	DD-Ct (mean)
Wild Type - cholesterol non fed	1

Wild Type- cholesterol fed	0.073
NOX5 knockout -cholesterol non fed	0.479
NOX5 knockout - cholesterol fed	0.005

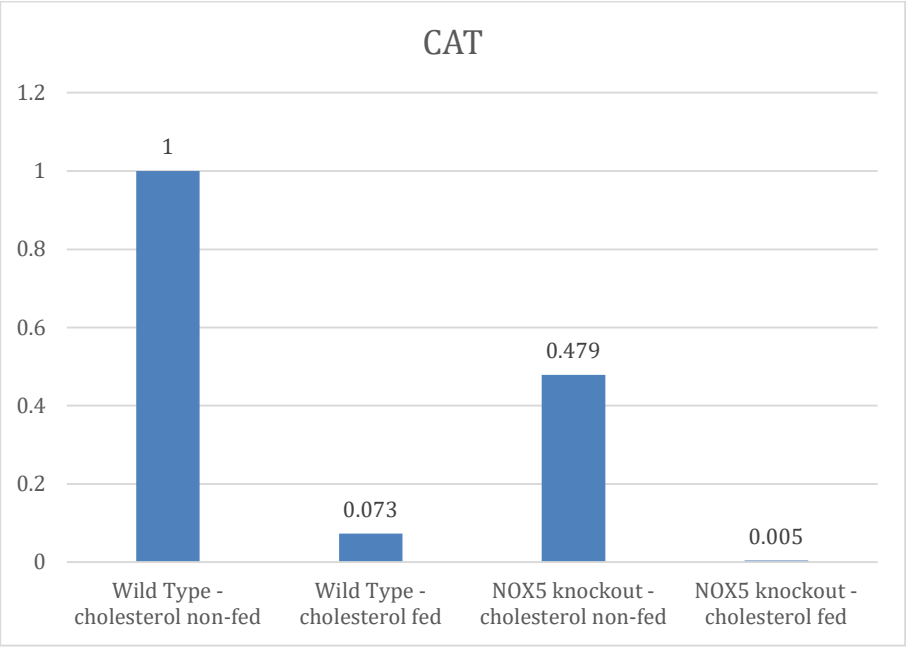

Figure 14: Bar graph for relative gene expression of *CAT*

Table 8 shows the values of relative gene expression of *CAT* in the different samples, which are visually represented in Figure 14. Upon analysis, similar trends were observed for the *CAT* gene, with wild-type rabbits on a cholesterol rich diet showing reduced *CAT* expression. *NOX5*-/- rabbits demonstrated a differential response in *CAT* expression levels under varying dietary conditions, with a slight increase observed in the standard diet group compared to a significant reduction under high cholesterol.

Wild-type rabbits on cholesterol-rich diet displayed a significant decrease in *CAT* expression to 0.073, suggesting a strong downregulation. *NOX5*-/- rabbits on standard diet exhibited a moderate decrease in *CAT* expression (Relative Expression: 0.479) compared to the wild-type baseline, indicating a possible adjustment to the absence of *NOX5*, though not as pronounced as seen in other antioxidant genes. *NOX5*-/- Rabbits on Cholesterol Rich Diet: showed an almost complete suppression of *CAT* expression (Relative Expression: 0.005), pointing to a profound impact of the combined effect of *NOX5* gene knockout and cholesterol rich diet.

The catalase gene plays a pivotal role in cellular antioxidant defense by catalyzing the decomposition of hydrogen peroxide, a harmful by-product of cellular metabolism, into water and oxygen. The decrease in *CAT* expression in wild-type rabbits fed a cholesterol rich diet underscores the detrimental impact of such a diet on oxidative stress. This reduction in *CAT* expression could contribute to an enhanced oxidative stress environment, increasing the development of atherosclerosis.

PRDX (Peroxiredoxin) **Gene Expression**

Table 9: *PRDX* Gene Expression

NOX5	DD-Ct (mean)
Wild Type - cholesterol non fed	1
Wild Type-cholesterol fed	0.066
NOX5 knockout -cholesterol non fed	2.405
NOX5 knockout -cholesterol fed	0.050

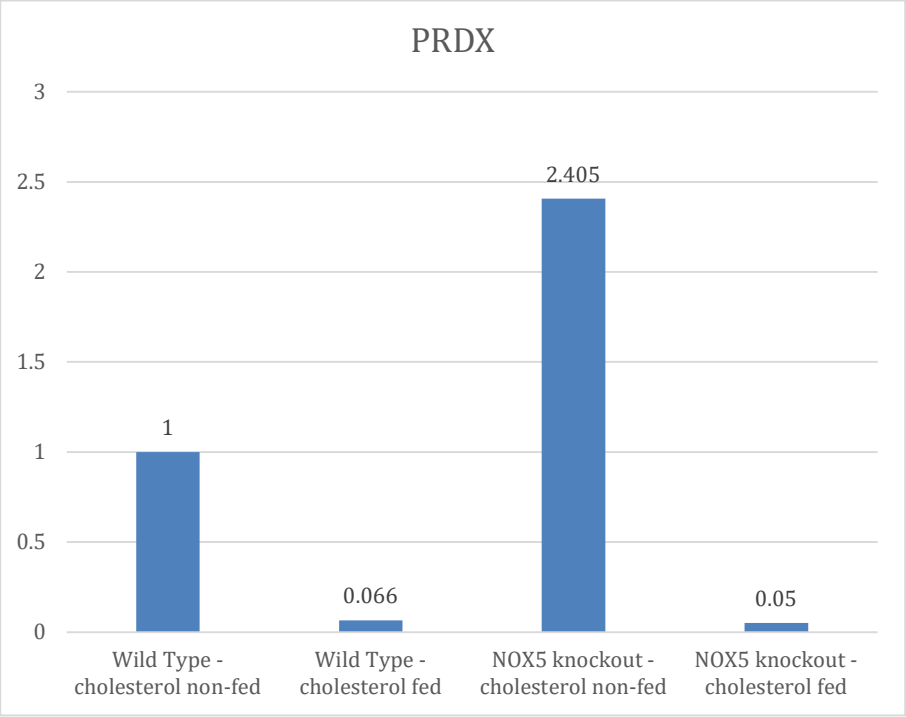

Figure 15: Bar graph for relative gene expression of *PRDX*

In Wild-type rabbits on cholesterol rich diet the expression of *PRDX* decreased to 0.067 in response to the cholesterol rich diet, indicating that elevated cholesterol levels might suppress *PRDX* expression. *NOX5*-/- Rabbits on standard diet showed an over twofold increase in *PRDX* expression (Relative Expression: 2.405), suggesting a potential interaction with *NOX5*. *NOX5*-/- rabbits on cholesterol rich diet, similar to the trend observed with *SOD3* and *CAT*, *PRDX* expression was further suppressed under cholesterol rich conditions (Relative Expression: 0.050), emphasizing the impact of diet on antioxidant gene expression. Table 9 shows the values of relative gene expression of *PRDX* in the different samples, which are visually represented in Figure 15.

NOX5 Gene Expression

Table 10: *NOX5* Gene Expression

NOX-5	DD-Ct (mean)
Wild Type - cholesterol non fed	1
Wild Type-cholesterol fed	0.401
NoX5 knockout -cholesterol non fed	0.330
NoX5 knockout –cholesterol fed	0.002

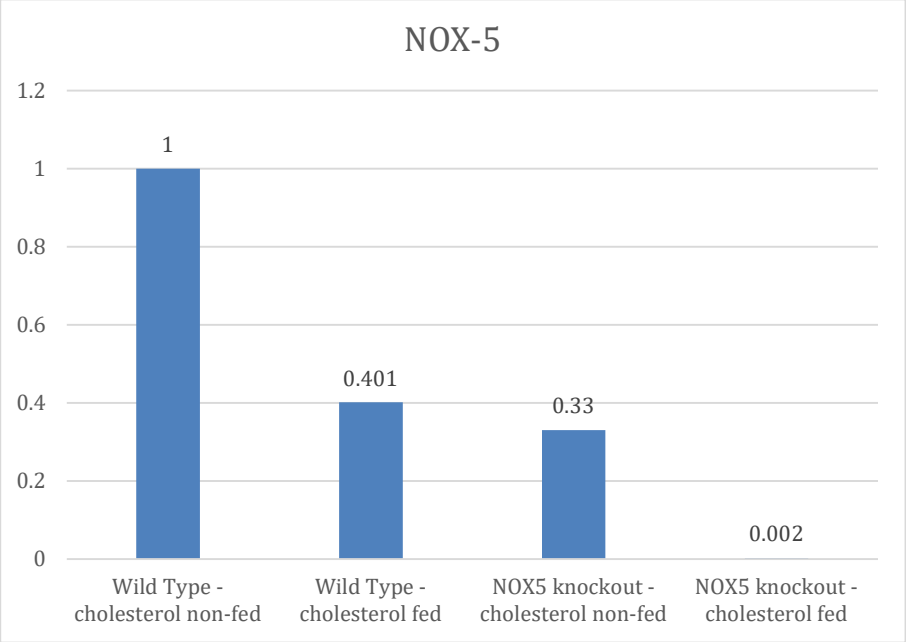

Figure 16: Bar graph for relative gene expression of NOX-5

Wild-type rabbits on cholesterol rich diets exhibited a moderate decrease in *NOX5* expression (Relative Expression: 0.401), suggesting that a cholesterol rich diet may influence *NOX5* expression levels. *NOX5*-/- rabbits on Standard Diet as expected showed a reduction in *NOX5* expression (Relative Expression: 0.330), indicating the genetic modification's effect. However, the expression did not completely drop to zero, possibly due to residual expressions. A residual expression of the gene in knock-out animals is a well-described phenomenon, as the mutation that causes an early stop-codon results in the lack of a functional protein, however on *RNA* level it can still be detected. Usually, the efficiency of transcription is much lower, and the defective *RNA* is under continuous degradation, so its expression level is usually lower compared to the wild type, just as we observed in our experiment. Table 9 and Figure 16 show the relative gene expression of *NOX5* gene. *NOX5*-/- rabbits on a cholesterol-rich diet showed a drastic reduction in *NOX5* expression to nearly undetectable levels (Relative Expression: 0.002).

Knockout – Wildtype Comparison

Table 11: Comparison of knockout and wild-type rabbits

Gene	Diet Condition	Expression in WT (DD-Ct Mean)	Expression in KO (DD-Ct Mean)	Observation
SOD3	Standard Diet	1	5.259	KO animals show a compensatory increase in *SOD3* expression.
SOD3	Cholesterol Rich Diet	0.094	0.081	Both WT and KO animals experience suppressed *SOD3* expression under a cholesterol rich diet.
CAT	Standard Diet	1	0.479	KO animals exhibit a moderate decrease in *CAT* expression.
CAT	Cholesterol Rich Diet	0.074	0.005	Cholesterol rich diets reduce *CAT* expression, especially in KO animals.
PRDX	Standard Diet	1	2.405	KO animals show increased in *PRDX* expression.
PRDX	Cholesterol Rich Diet	0.067	0.05	Both groups show reduced *PRDX* expression under a Cholesterol rich diet, but the effect is less pronounced.
NOX5	Standard Diet	1	0.33	Reduced *NOX5* expression in KO animals confirms the effectiveness of the knockout.

It is observed that under standard diet conditions, KO animals demonstrate an elevated expression of antioxidant enzymes such as *SOD3* and *PRDX.* This increase might be a response to the absence of *NOX5*, however the underlying molecular mechanism needs to be further investigated. Both wild-type (WT) and KO animals subjected to a cholesterol-rich diet exhibited suppressed expression of key antioxidant enzymes, including *SOD3*, *CAT and PRDX*

in our experiment. This suppression highlights the influence of diet on the body's ability to manage oxidative stress, demonstrating that a cholesterol rich diet can negatively effect on the long run the gene expression related to antioxidant defenses across genetic variations. Particularly the reduction in *CAT* expression observed in KO animals on a cholesterol rich diet. This near-complete suppression of *CAT* expression points to a vulnerability in animals lacking *NOX5* when exposed to dietary conditions that exacerbate oxidative stress. It suggests that the absence of *NOX5*, compounded by a cholesterol rich diet, leaves the organism exceedingly susceptible to oxidative damage, underlining the critical role of *NOX5* in maintaining oxidative balance under stress-inducing dietary conditions. These results support our research group's previous finding that *NOX5* -KO genotype increases the atherosclerotic effect of cholesterol-rich diet (Petheő, 2021.) As anticipated, the expression of *NOX5* is inherently reduced in KO animals. However, the introduction of a cholesterol rich diet further diminishes its expression to nearly undetectable levels, particularly in KO animals. This observation aligns with expectations, considering the genetic deletion of *NOX5*, but also emphasizes the impact of dietary factors on the expression of genes involved in oxidative stress and antioxidant defense mechanisms.

5. DISCUSSION

The comparison between RNAzol and TRIzol revealed that the TRIzol generally produced higher purity *RNA*, despite yielding a wider range of *RNA* concentrations. However, both methods showed contamination issues, particularly with organic compounds. This underscores the need for further optimization of *RNA* isolation methods to improve both quality and yield. The finding of a study explains that modifying steps in the *RNA* isolation process using the TRIzol reagent kit led to improved *RNA* purification (Zhang et al., 2023).

The outcome of this thesis has provided insight into the relative expression of antioxidant genes (*CAT, SOD3, PRDX*) and the oxidative stress-related gene *NOX5* in wild-type and *NOX5*-/- rabbits under varying dietary conditions reveals some insights into the dynamics of oxidative stress management and cardiovascular health. This thesis indicated that a cholesterol-rich diet suppresses *SOD3* expression in both wild-type and *NOX5*-/- rabbits. This reduction in *SOD3* suggests that dietary cholesterol negatively impacts the cellular antioxidant capacity. The observed decrease in *SOD3* expression aligns with previous studies suggesting that high cholesterol levels can impair antioxidant defenses, potentially exacerbating oxidative stress and contributing to the development of cardiovascular diseases (CVDs) (Zelko et al., 2002; Rykova et al., 2019). An increase in *SOD3* expression among standard diet-fed NOX5-/- rabbits suggests an adaptive mechanism to counterbalance the absence of *NOX5.*

The expression levels of *CAT* and *PRDX* in our study were impacted by both the genetic background (*NOX5* deficiency) and dietary cholesterol. This finding is particularly concerning for *NOX5*-/- rabbits, which exhibited almost complete suppression of *CAT* expression, suggesting impairment in their ability to neutralize reactive oxygen species (ROS). This points out the importance of antioxidant defenses in maintaining cardiovascular health (Bedard & Krause, 2007; Kumar et al., 2010).

The decrease in *NOX5* expression in wild-type rabbits fed a cholesterol rich diet and in *NOX5*-/- rabbits, further explains the relationship between diet, gene expression, and oxidative stress. *NOX5* is known to contribute to ROS production; thus, its modulation by dietary cholesterol suggests a possible regulatory mechanism through which diet influences oxidative stress levels (Kattoor et al., 2017; Rivera et al., 2010). However, the almost undetectable levels of *NOX5* expression in *NOX5*-/- rabbits on a cholesterol rich diet indicate a substantial

alteration in the oxidative stress which could potentially increase susceptibility to oxidative damage and atherosclerosis.

The findings from this thesis align with and extend the existing body of literature on the relationship between oxidative stress, antioxidant defenses, and cardiovascular health. Previous research has demonstrated the crucial role of *SOD3* in protecting against vascular diseases and its potential as a therapeutic target for mitigating oxidative stress-related pathologies (Rykova et al., 2019; Zelko et al., 2002). This contributes to this knowledge base by elucidating the effects of dietary and genetic factors on *SOD3* expression, offering insights into the complex mechanisms governing cardiovascular disease development and progression.

The suppression of *PRDX* and *NOX5* expression in *NOX5*-/- rabbits under a cholesterol rich diet highlights the crucial role of dietary factors in modulating gene expression. These findings align with previous studies indicating the importance of NADPH oxidases in cardiovascular diseases and their regulation by dietary and genetic factors (Cave et al., 2006; Schulze & Lee, 2005). Specifically, the role of NOX enzymes in generating reactive oxygen species and their involvement in atherosclerosis development is well documented, with dietary cholesterol being a known modulator of their expression and activity (Kattoor et al., 2017; Rivera et al., 2010).

The upregulation of *PRDX* in *NOX5*-/- rabbits on a standard diet suggests that alternative pathways might be activated to maintain oxidative balance in the absence of *NOX5*, a hypothesis supported by the study's findings and literature on oxidative stress responses (Dikalov, 2011; Holterman et al., 2014). However, the reduction in *PRDX* and *NOX5* expression under cholesterol-rich conditions in *NOX5*-/- rabbits could exacerbate oxidative stress, potentially contributing to the accelerated development of atherosclerosis observed in these models.

6. CONCLUSIONS AND RECOMMENDATIONS

This thesis successfully identified how *NOX5* interacts with a cholesterol-rich diet to influence oxidative stress and antioxidant defenses in rabbits with the help of Nox5 knockout rabbit model. We found that dietary cholesterol suppresses the expression of key antioxidant genes such as *SOD3*, *CAT*, and *PRDX*, highlighting the negative impact of high cholesterol on cellular antioxidant capabilities, potentially worsening cardiovascular diseases. The study showed that the absence of *NOX5* amplifies these effects, indicating a crucial role for *NOX5* in managing oxidative stress under dietary challenges. These insights emphasize the interconnection between genetics and dietary factors in cardiovascular health.

Based on these findings, it is recommended to extend research into the molecular mechanisms of *NOX5* and its interaction with dietary components. Future studies should explore the chronic effects of dietary cholesterol across different models and longer-term studies to better understand its impacts on cardiovascular health. Additionally, expanding genetic studies and exploring therapeutic targets within the *NOX5* pathways could lead to novel interventions for mitigating the adverse effects of high cholesterol. The ultimate goal would be to translate these findings into practical dietary recommendations and therapeutic strategies that address the cardiovascular risks associated with dietary choices and genetic predispositions.

Our experiments involving RNA isolation faced technical difficulties that significantly impacted the results of quantitative PCR. To achieve more accurate results, it is essential to optimize the RNA isolation process by possibly adopting a more advanced isolation kit and accounting for the high-fat content of brain tissue.

In future research, a robust quality control protocol, such as verifying RNA purity on an agarose gel or using a Bioagilent analyzer, should be implemented. Therefore, the findings of this thesis should be approached with caution.

7. SUMMARY

Cardiovascular diseases (CVDs) are one of the major global health concerns that are largely caused due to oxidative stress, which plays a crucial role in their pathogenesis by causing an imbalance between reactive oxygen species (ROS) production and antioxidant defenses. This study explores the role of *NOX5*, a member of the NADPH oxidase family that produces ROS in response to calcium signals, in cardiovascular health. It investigates how *NOX5's* interaction with dietary cholesterol affects the development of cardiovascular diseases, particularly focusing on its role in oxidative stress in rabbit models. By examining both wild-type and *NOX5*-knock out rabbits under varying cholesterol diets, this study aims to understand how *NOX5* expression and activity influence key antioxidant genes in the brain.

The study utilized two groups: *NOX5*-knockout and wild-type rabbits, both aged 20 weeks, subjected to controlled environments and dietary regimens (cholesterol fed and unfed). For sample collection, a humane euthanasia method was employed, followed by rapid sample preservation to ensure *RNA* integrity for subsequent gene expression analyses. The study employed both RNAzol and TRIzol reagents for *RNA* isolation, comparing their efficiency in preserving *RNA* quality, which was critical for accurate downstream qPCR analysis. The study compared the efficacy of RNAzol and TRIzol methods for *RNA* isolation, using Nanodrop spectrophotometry to evaluate the purity and concentration of *RNA*. The TRIzol method demonstrated superior purity, indicated by more favorable A260/A230 absorbance ratios, suggesting less phenol contamination compared to RNAzol. However, the RNAzol method often resulted in higher *RNA* concentrations. This suggests that while TRIzol may be more suitable for high-purity applications, RNAzol could be considered when higher *RNA* yield is required despite potential purity concerns.

Melting curve analysis was used to test the primers. It is important for assessing the specificity and efficiency of primer pairs used in gene expression studies. This analysis indicated that the primer pairs for *SOD3*, *CAT*, and *PRDX* showed specific amplification, indicating they are suitable for further research. However, the primer pairs for *NOX5* demonstrated non-specific amplification and primer-dimer formation, highlighting the need for their redesign or optimization.

Quantitative Real-Time PCR (q-PCR) was utilized to measure the expression of genes associated with oxidative stress and antioxidant defenses *(SOD3, CAT, PRDX, NOX5*) in both *NOX5*-deficient and wild-type rabbits subjected to standard and cholesterol rich diets. The findings indicated a decrease in *SOD3* expression in wild-type rabbits on a cholesterol rich diet, highlighting the effects of dietary cholesterol on this antioxidant mechanism. Conversely, *NOX5* -deficient rabbits on a standard diet showed an increase in *SOD3* expression, suggesting a potential compensatory mechanism for the absence of *NOX5*, though this effect was lost under high-cholesterol conditions. *CAT* and *PRDX* expressions followed similar patterns, with significant downregulation in both genes under cholesterol-rich diets in all rabbits. This suppression was more pronounced in *NOX5*-deficient rabbits, suggesting the impact of high cholesterol on crucial antioxidant defenses.

In conclusion, the results hypothesized that a cholesterol-rich diet suppresses the expression of key antioxidant genes, leading to an increase in oxidative stress, especially in the absence of *NOX5*. This emphasizes the need for further research to explore the molecular mechanisms through which *NOX5* interacts with dietary cholesterol, aiming to develop effective interventions that could mitigate the cardiovascular risks associated with high-cholesterol diets. Future studies should extend to different models and longer-term dietary assessments to fully understand the correlation between genetics, diet, and cardiovascular health.

Technical difficulties arise during the experiments with *RNA* isolation that have a major effect on quantitative PCR results. Further optimization of *RNA* isolation will lead to more accurate results. A more study quality control of *RNA* purity must be included in the research pipeline in the future.

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