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THESIS

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GENE EXPRESSION CHANGE OF OXIDANT AND ANTIOXIDANT SYSTEM DUE TO HIGH CHOLESTEROL DIET IN NOX-4 KNOCK OUT RABBIT

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Abstract

MUHAMMAD FARHAN. Gene Expression Changes Of The Oxidant and Antioxidant System Due To High Cholesterol Diet In Nox4 Knock-Out Rabbit**.** Supervised by BODROGI LILLA and TIMEA PINTER.

The role of NADPH oxidase enzymes in the regulation of many physiological functions is still not fully understood. The use of NOX inhibitors may help to highlight how NOX regulates these pathways and NOX inhibitors have also a potential therapeutic purpose. Indeed, while the physiological activity of NOX is crucial for health, oxidative stress due to excessive activity of NOX may lead to pathology. The aim of this study was to better understand the role of one of the isoforms of the NOX family of enzymes, NOX4 in the pathogenesis of atherosclerosis by the investigation of the gene expression changes of the oxidant and antioxidant system in response to high cholesterol diet in Nox4 knock-out rabbits. The rabbits were divided into two groups: one fed a high cholesterol diet and the other a normal diet. Gene expression analysis of the oxidant and antioxidant system was performed using real-time PCR. The results showed that the high cholesterol diet led to a significant increase in the expression of catalase in wild type animals and the increase of PRDX in the Nox4 knock-out group. In contrast, the expression of Nrf2 and Gpx1 genes were significantly decreased in both groups, but the Nrf2 expression was higher in the Nox4 knock-out group. These findings suggest that Nox4 knock-out may enhance the antioxidant defense system in response to a high cholesterol diet, potentially providing a protective effect against oxidative stress. Further studies are warranted to elucidate the underlying mechanisms of these effects.

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List of Abbreviations

AML	Amyotrophic Lateral Sclerosis
AP-1	Activator Protein 1
ApoE	Apolipoprotein E
AR2202CGD	Autosomal Recessive Forms of Chronic Granulomatous Disease
ARE	Antioxidant Response Element
ATP	Adenosine Tri Phosphate
bFGF	Basic Fibroblast Growth Factor
BMI	Body Mass Index
Bp	Base Pair
CA	Coronary Atherosclerosis
CAD	Coronary Artery Disease
Cas9	CRISPR-associated protein 9
CAT	Catalase
cDNA	Complementary Deoxyribonucleic Acid
COVID-19	Coronavirus disease 2019
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle Threshold
CVD	Cardiovascular Disease
CVE	Cardiovascular Events
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
DUOX	Dual Oxidase

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EC	Endothelial Cells
eNOS	endothelial Nitric Oxygen Species
G3P	Glyceral-3-Phospahte
GADPH	Glyceraldehyde 3-Phosphate Dehydrogenase
GM	Genetically Modified
GMO	Genetically Modified Organism
GOI	Gene of Interest
GPX	Glutathione Peroxidase
GSSG	Glutathione disulfide
HO-1	Heme Oxygenase 1
ICAD	Intracranial Atherosclerotic Disease
IFN	Interferon
IH	Ischemic Heart
ILL-1	Interleukin 1
iPSC	Induced Pluripotent Stem Cell
IS	Ischemic Stroke
Keap1	Kelch-like protein 1
KO	Knock Out
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LPS	Lipopolysaccharide
Ly6C	Lymphocyte Antigen 6 Complex
mAbs	monoclonal antibodies
MI	Myocardial Infarction

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mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH Oxidase
NQO1	NAD(P)H Quinone Dehydrogenase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OxLDL	Oxidized Low Density Lipoprotein
pAbs	Polyclonal Antibodies
PAD	Peripheral Arterial Disease
PCR	Polymerase Chain Reaction
PRDX	Peroxiredoxin
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT PCR	Real Time Polymerase Chain Reaction
sgRNA	Single Guide Ribonucleic Acid
SMC	Smooth Muscle Cell
SOD	Superoxidase dismutase
STAT6	Signal Transducer and Activator of Transcription 6
TBI	Traumatic Brain Injury
TNF-α	Tumor Necrosis Alpha
VSMC	Vascular Smooth Muscle Cell
X0CGD	X-linked Chronic Granulomatous Disease

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XO Xanthine Oxidase

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Introduction

Cardiovascular disease (CVD), mainly ischemic heart disease (IH) and stroke are major causes of global mortality and contribute to disability. CVD is also a major cause of the increasing health care costs and becoming a global burden. The burden of CVD continues to rise globally in number of deaths, years of life lost, and disability-adjusted life years. From 1990 to 2019, the prevalent case of CVD had nearly doubled, and the mortality of CVD had steadily continued to increase. (Roth *et al.* 2020). The Global Burden of Cardiovascular Disease Study has identified leading risk factor of cardiovascular disease death: high blood pressure, dietary risk, low density lipoprotein cholesterol (LDL-C), air pollution, tobacco smoking and secondhand tobacco smoke, high body mass index (BMI), high fasting plasma glucose closely related to diabetes, kidney dysfunction, lead exposure, non-optimal temperature, low physical activity, high alcohol consumption, and COVID-19 (Vaduganathan 2022).

Dyslipidemia, smoking, and hypertension are found to be the most frequent risk factors (RF) for cardiovascular events (CVEs) in the 15 cities young stroke study in Europe. Several European long-term young stroke studies have shown recurrent of CVEs, such as myocardial infarction (MI), angina, peripheral arterial disease (PAD), ischemic stroke (IS), and mortality mainly caused by coronary atherosclerosis (CA). Atherosclerosis is highly prevalent in young stroke patients and even in control patients showing that CVEs are only 'the tip of iceberg' and clinical and subclinical atherosclerosis may start early in life (Nawaz *et al.* 2021). Symptomatic intracranial atherosclerotic disease (ICAD) is associated with early recurrence of ischemic stroke. Intracranial atherosclerotic plaque rupture or intraplaque hemorrhage and thrombus formation is thought to be mechanism of stroke in ICAD (Yaghi *et al.* 2019).

The term atherosclerosis is from Greek language, 'athere' meaning gruel or lipid accumulation and 'sclerosis' meaning hardening. Atherosclerosis is characterized by cholesterol accumulation, macrophages infiltration, smooth muscle cells (SMC) proliferation, connective tissue components accumulation, and thrombus formation. Hyperlipidemic state, smoking, stress, and hypertension are some of the risk factors of atherosclerosis (Singh *et al.* 2002). Atherosclerosis lesion develops in the intima layer of the arterial wall. Lesion development is accompanied by local increase of macrophages and hematogenous cells. Pericyte-like cells,

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macrophages, and some smooth muscle cells accumulate lipids and turn it into foam cells. The primary source of accumulated lipids is low density lipoprotein (LDL). LDL is susceptible to modifications. Atherogenic modification of LDL starts with desialylation, continued by changes of lipoprotein in size, density, and electric charge resulting in small dense and electronegative LDL fraction. The interaction of the immune system and LDL induces formation of foam cells. The primary event of atherosclerotic lesion development is local endothelial activation and permeability increase. Endothelial activation express cytokines and chemokines (interleukin 1 [ILL-1]), tumor necrosis alpha (TNF-α), chemokines monocyte chemoattractant (MCP-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and adhesion molecules. Thus, resulting in endothelium interaction increase and penetration into the subendothelial space. This violation of immune response causes inflammation chronification and leads to atherosclerotic lesion development (Markin *et al*. 2020). Increased generation of reactive oxygen species (ROS), especially superoxide is associated with endothelial dysfunction. Superoxide is well studied to have an important role as signaling molecules in normal vascular function, but excess production of ROS is highly pathological and associated with atherogenesis by damaging proteins, lipids, and NO from the blood vessel wall (Douglas and Channon 2014).

Excessive production of ROS outpacing the capability of antioxidant system generates oxidative stress. Several ROS-producing systems are present in blood vessels, such as xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS), mitochondrial respiratory chain enzymes, and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX). Oxidative stress has been studied and shown to be an important factor in atherogenesis. These conditions induce cell injury by directly oxidizing and damaging cellular proteins, lipid, and DNA (Yang *et al*. 2017).

However, unlike NOX1 and NOX2 which have been proven in the development of atherosclerosis and other related CVDs, the role of NOX4 remains unclear and uncertain in CVD pathogenesis, including atherosclerosis pathogenesis. NOX4 was suggested to have vasoprotective role in atherosclerosis (Fulton and Barman 2016; Schroder *et al.* 2012), while NOX4 was also suggested to upregulate the migration of SMCs and lead neointimal plaque formation through specific activation (Kim *et al.* 2019).

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The present study is a part of a broader research program to preserve a better and concrete understanding of NOX4 role in cardiovascular diseases and other related degenerative diseases. This study particularly focused on the gene expression analysis of four antioxidant genes in Nox4 knock out (KO) rabbits. Previously, NOX4 KO rabbit line was successfully generated and bred to establish a homozygous population of animals.

Question

How does high cholesterol affect the gene expression profile of antioxidant system in NOX4 knock-out rabbits?

Objectives

The present study is a part of a broader research program to preserve a better and concrete understanding of NOX4 role in cardiovascular diseases and other related degenerative diseases. This study particularly focused on the gene expression analysis of four antioxidant genes in Nox4 knock out (KO) rabbits. Previously, NOX4 KO rabbit line was successfully generated and bred to establish a homozygous population of animals.

Experimental groups of Nox4-KO and wild type male rabbits were fed with cholesterol enriched diet (0,5 w/e) for 8 weeks. Blood samples were taken twice, at the start of the experiment (week 0) and after 8 weeks of feeding with cholesterol rich diet. Total RNA was extracted, and cDNA was synthetized. Lastly, gene expression analysis was carried out using qPCR of the synthesized cDNA.

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Literature Review

Reactive oxygen species

Reactive oxygen species (ROS) are reactive molecules and free radicals containing oxygen, such as superoxide anion (O2•-), hydrogen peroxide (H2O2), hydroxyl radical (•OH) and other common reactive oxygen species shown in Figure 1. Most ROS are formed naturally as byproducts of mitochondrial electron of aerobic respiration metabolism, produced by oxidoreductase enzymes and metal catalyzed oxidation (Zorov, Juhaszova, Sollott 2014). ROS also can be generated in response to various exogenous stimuli such as UV radiation, air pollution, environmental toxins, and smoking (Tavassolifar *et al.* 2020). ROS plays important roles in various cellular processes such as signaling, gene expression, and host defense against pathogens. However, excessive ROS production can lead to oxidative damage to cellular components such as DNA, proteins, and lipids, which has been implicated in the pathogenesis of various diseases including cancer, cardiovascular disease, and neurodegenerative disorders (Saha *et al.* 2017; Dubois-Deruy 2020; Boas, Joyce and Cowell 2021). Source of reactive oxygen species is rich under physiological and pathological conditions as well. Cells distribute multiple sources ROS, including mitochondria, endoplasmic reticulum, peroxisomes, NAD(P)H oxidases, and monoamine oxidases (Yu and Xiao 2021). Mitochondria acts as a major ROS producer since oxidative phosphorylation produces an incomplete oxygen reduction (Sharafi-Rad *et al.* 2020).

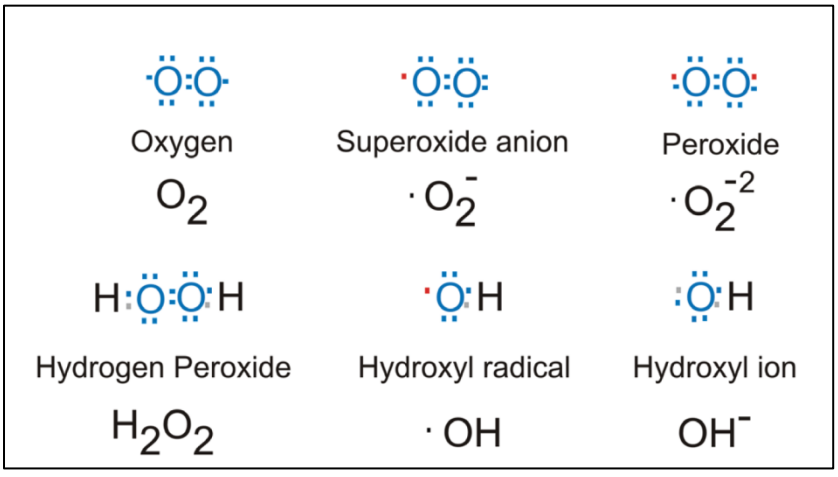

Figure 1. Lewis structure of common reactive oxygen species

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The generation and scavenging of ROS are tightly regulated by various enzymatic and non-enzymatic mechanisms in cells. Enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) play crucial roles in the detoxification of ROS (Ighodaro and Akinloye 2018). Non-enzymatic antioxidants such as vitamins C and E, glutathione, and carotenoids also play important roles in scavenging ROS (Kurutas 2016). Dysregulation of ROS production and antioxidant defense mechanisms can result in oxidative stress, which is characterized by an imbalance between ROS production and antioxidant capacity (Schieber and Chandel 2014).

ROS plays a crucial role in cellular signaling and regulation. Low levels of ROS can act as second messengers and activate signaling pathways involved in cell proliferation, differentiation, and apoptosis (Sinenko *et al* 2021; Milkovic *et al.* 2019; Circu and Aw 2010). ROS are also able to modulate the activity of transcription factors such as NF-κB, AP-1, and Nrf2, which regulate the expression of genes involved in stress response and antioxidant defense (Lingappan 2018; Shi and Gibson 2007; Hiebert 2021).

Oxidative stress and atherosclerosis

Atherosclerosis is a disease characterized by the accumulation of lipids, fibrous elements, and calcification within the arteries. Its development starts with endothelial cell (EC) activation, followed by several signaling pathways leading to plaque buildup within the arteries, narrowing of the blood vessels and eventually resulting in an increased risk of cardiovascular disease (Jebari-binslaiman 2022). Atherosclerosis is initiated by endothelial dysfunction with low density lipoprotein (LDL) retention and its modification in the intima (Hermadi N *et al.* 2014; Mundi S, *et al.* 2018).

Both hyperlipidemia and oxidative stress are thought to play a role in the development and progression of atherosclerosis. Lipid overload increases lipopolysaccharides (LPS) circulating levels and oxidative stress. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense and causes lipoprotein oxidation which is involved in atherosclerosis pathogenesis and changing cellular function. Oxidized low density lipoprotein (oxLDL) are proven to induce several proatherogenic processes, including oxidative burst modulation, dendritic cells maturation, alternative (M2)

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macrophage activation, T2 helper response [Figure 2] that involved in plaque development (Peluso *et al.* 2012).

NAPDH oxidases (NOX) are probably the most important ROS generating system in the cardiovascular system. NOX activity induces expression of vascular smooth muscle cell (VSMC) proliferation, endothelial adhesion, and monocytes infiltration, Activation of NOX generates superoxide anion by transferring electron from NADPH to oxygen molecule (Kattoor *et al.* 2017).

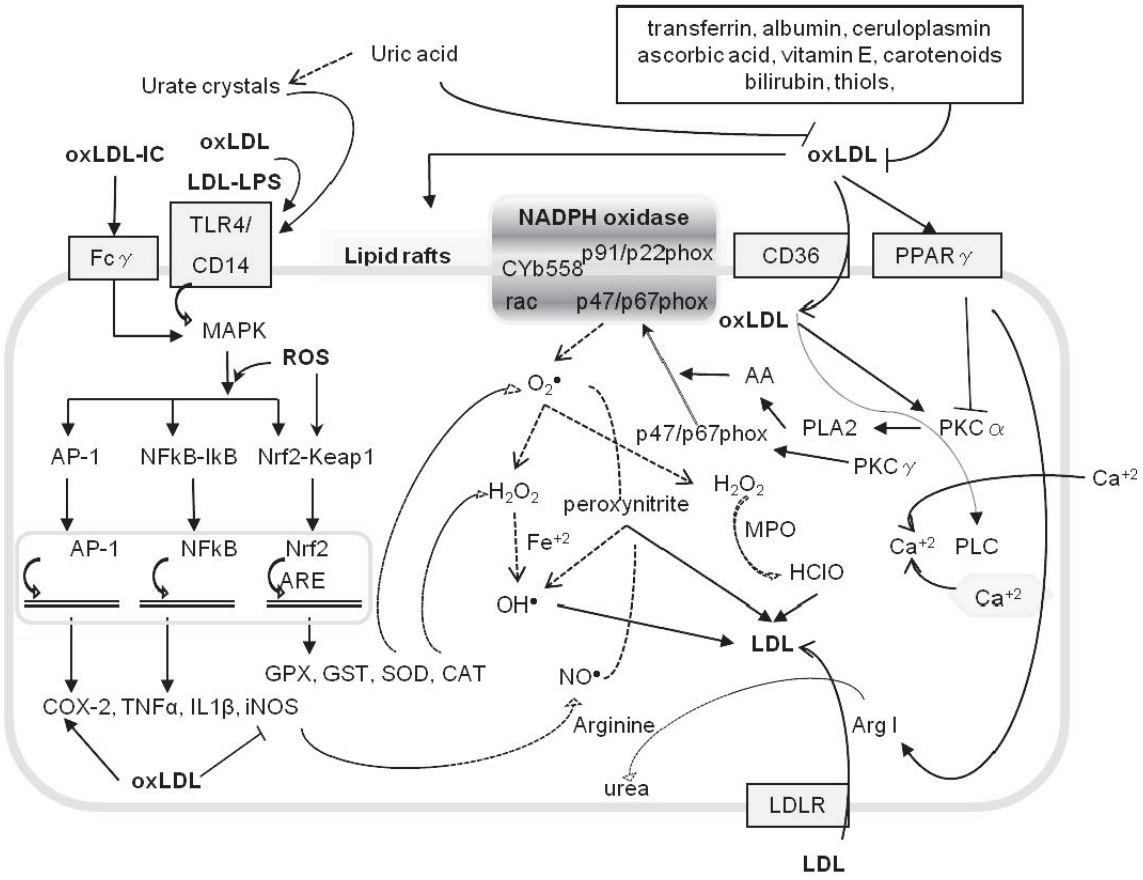

Figure 2. Molecular and cellular regulation of redox status and oxidative burst during the interaction of LDL and leukocytes (Peluso *et al.* 2012).

Excessive ROS also contributes to the development of atherosclerosis by damaging the molecules that make up the walls of the arteries and cause inflammation (Chen *et al.* 2018). This can lead to the formation of plaque, which is made up of cell lesions, foam cells, cholesterol ester, calcium, and a mass of fatty acids that accumulate in the arteries (Rafieian-Kopaei 2014). Atherosclerotic

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plaque can narrow the arteries and make it difficult for blood to flow through them, increasing the risk of heart attack and stroke (Arboix 2015).

NADPH oxidases

There are several different types of NADPH oxidases, which are found in different cell types and play different roles in the body. Humans have a family member of seven different enzymatic isoforms, NOX1-5 and DUOX1-2. In general, NADPH oxidases are involved in the production of ROS in response to various stimuli, such as inflammation, stress, and immune system activation (Buvelot *et al.* 2019). NADPH oxidases reduce oxygen to superoxide radical anion (O_2^{2-}) by using NADPH as an electron donor according to reaction below:

$$NADPH + O_2 \longrightarrow NADP^+ + 2O_2^{2-} + H^+$$

ROS produced by NADPH oxidases have very diverse functions, both beneficial and harmful effects on the body. On the one hand, ROS produced by NADPH oxidases play important roles in cell signaling and in the immune system against microorganism through oxidative burst. They can act as signaling molecules to regulate various cellular processes, such as endothelial signaling, serotonin biosynthesis, regulation of renal function, and cellular proliferation. But their overexpression is also associated with various neurological diseases and types of cancer (Skonieczina *et al.* 2017).

On the contrary, ROS produced by NADPH oxidases also play an important role in the pathogenesis of several neurodegenerative diseases and neuroinflammation. NOX activation in the hippocampus has been associated with cognitive damage followed by cerebral ischemia and hippocampal cell death (Tarafdar and Pula 2018). NOX also cause damage and further injury in traumatic brain injury (TBI), stroke, amyotrophic lateral sclerosis (AML) (Rastogi 2017). In diabetic melitus patients, hyperglycemia condition induces ROS production increase by NOX enzymes and believed to play a pivotal role in cardiovascular dysfunction ((Lu *et al.* 2020; Teshima *et al.* 2014; Wang *et al.* 2021).

In induced pluripotent stem cells (iPSCs) derived from X-linked Chronic Granulomatous Disease (X0CGD) patients with NOX2 deficiency, and AR220CGD patients with p22phox subunit deficiency which reduces NOX1, NOX2, NOX3 and NOX4 activity. NOX4 is the main NOX enzyme which is engaged in the initial phases of iPSC-derived hematopoietic development

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and its activity can influence the amount, hematopoietic potential, and phenotype of CD34+ (Brault *et al.* 2019).

The Nox4 deficit drives proinflammatory macrophage M(LPS+IFN) and lowers wound-healing macrophage M(IL4+IL13) polarization. In comparison to wild type M(LPS+IFN)-polarized macrophages, Nox4-/- macrophages express more Nox2 and generate more superoxide anions. The greater level of Nox2 in Nox4-deficient M(LPS+IFN)-polarized macrophages is caused by the increased NFκB activity, which is caused by the reduced STAT6 activation and increased NFκB activity that Nox4 deficiency causes. According to those findings, Nox4 loss induces the production of proinflammatory genes and cytokines in vivo in a murine inflammation-driven fibrosarcoma model, along with an increase in the quantity of proinflammatory Ly6C+ macrophages in the tumors. Overall, the results of this investigation point to Nox4 having anti-inflammatory properties in macrophages. Less M(IL4 + IL13) polarization and NFκB activity in monocytes are caused by a Nox4 deficit (Helfinger *et al.* 2019).

GMO animals in NADPH-oxidase research

Genetically modified (GM) animals are animal whose genetic material has been artificially altered by transgenic technologies leading t deletion, insertion, and/or substitution of part of the genome Thus, genetic studies have been important to biomedical research, attempt to understand illnesses and diseases and way to cure, prevent, and treat them. Animals are good systems to study gene function and physiological and pathological processes, however the translational value of an organism is highly dependent on species specific characteristics of its anatomy, physiology and genetic distance to human. With the possibility to introduce very precisely designed genetic changes to basically any mammalian species with modern genome editing technologies, the translational value of these animals is vast. Carefully designed stretches of DNA can be inserted to a random (classical additive transgenesis) or highly targeted sites in the genome this is the so-called knock-in technology, or knock-out of genesrender its function is also achievable in order to alter, repair or modify its function (Bailey 2019).

GM animals are or could be produced for diverse purposes including xenotransplantation, protein pharming, enhancement of production traits, or for basic research.. Xenotransplantation

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is cells, tissues, or organs transplantation between species. Pharming is a practice to produce biopharmaceuticals through transgenic animals with a cost-effective method. Animal enhancement is an approaches that aims animals to have better growth rates for example or better resistancy to certain diseases. Lastly, GM animals are used to better understand the mechanism genetic modification itself (Keane 2019).

In terms of genetics, rabbits share many of the same genes with humans, making them a valuable model for studying the effects of genetic mutations and potential treatments. Additionally, rabbits have similar cardiovascular and respiratory systems, as well as digestive and immune systems, which are relevant for studying diseases and use the animals in drug development. Moreover, rabbits are widely used in research on wound healing and tissue regeneration, as their skin and bone structures are similar to humans. Even though they are excellent experimental models for cardiovascular research, rabbits should not be mistaken for rodents. Only investigations into processes that cannot be adequately addressed in other animal models, such as translational research for the creation of lipid-lowering medications or diagnostic procedures, should be conducted on rabbits. Without taking these factors into account, utilizing rabbits may not be as advantageous as using rats, which have advantages over rabbits such as shorter gestation times, shorter life spans, reduced inter-individual variability of inbred strains, lower maintenance costs, and a wider range of reagent availability. Rabbits should thus be utilized to fill the gaps between small animal models (mice and rats), which are probably ideal for elucidating gene expression and functions, and larger animal models (pigs and monkeys), which are frequently needed for pre-clinical translational research. When compared to those larger animals, medium-sized rabbits may be kept in labs and are comparatively cheap to buy, house, and care for. They are simple to handle and breed, and the scientific and regulatory organizations have long recognized them as a good model. Also, because rabbits have a more diversified genetic background than inbred and outbred rodent strains and are phylogenetically closer to primates than rodents, the model is a better overall representation of humans (Fan *et al.* 2015; Baumgartner *et al.* 2016). Several studies have used rabbits as a translational research model to investigate human diseases and drug efficacy. For example, a study by Deci *et al*. (2020) used a rabbit model to investigate different pathological markers of Alzheimer's disease. The study showed further validation on the value of rabbit as an animal model of Alzheimer's disease. A review study by Mage, Esteves, & Rader (2019) presents utility of rabbits as a tool to develop

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diagnostics and therapeutics with examples from diagnosing and treating human disease with rabbit polyclonal and monoclonal antibodies (pAbs and mAbs, respectively), through studies of cardiovascular, autoimmune, ophthalmological, neoplastic diseases, and to new insights into the role of endogenous retroviruses.

The knock-out rabbit model used in our study was generated by knocking out the NOX4 gene, which is a member of the NOX family of enzymes involved in redox signaling and oxidative stress. The NOX4 KO rabbit lines were created using CRISPR/Cas9 system guided by gRNA designed to the second exon of NOX4 gene. It resulted in founder NOX4 KO animals with no signs of off-targets events (Pinter *et al.* 2020). For our studies homozygous population was established carrying a 7 bp deletion in the 2nd exon of rabbit NOX4 gene leading to an early stop codon in the coding sequence and because of this functional protein is not produced.

Finally, a NOX5 knock-out rabbit model was generated by knocking out the NOX5 gene, which is a member of the NOX family that is involved in calcium signaling and cellular differentiation. Unlike rodents, rabbits share genetically similar NOX5 gene. The NOX5 KO rabbit lines generated by CRISPR with sgRNA to exon 3 of the rabbit NOX5 gene resulting in disrupted NOX5. Interesting result shown by the NOX5 knock-out rabbits indicates the protective role of NOX5 in atherosclerosis. The NOX5 knock-out rabbits exhibited increased atherosclerosis in rabbits shown by more plaque formation in thoracic aortas (Petheő *et al.* 2021).

Antioxidant system

The antioxidant system is a network of proteins, and their interactions help to protect cells and tissues from damage caused by reactive oxygen species (ROS). Several key genes and proteins are involved in the antioxidant system, including Nrf2, a transcription factor which activate the expression of a number of genes involved in the antioxidant system under the regulation of antioxidant response elements including superoxide dismutase (SOD), NQO1, heme oxygenase-1 (HO-1), and glutathione peroxidase (GPX) to respond oxidative stress and maintain a balance redox state (Huang *et al*. 2015).

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Under normal conditions Kelch-like protein 1 (Keap1) binds to Nrf2 and promotes its degradation keeping Nrf2 levels low in the cytoplasm of the cell. When cells are exposed to oxidative stress (or other cellular stressors) Nrf2 is released from Keap1 and translocates to the nucleus where it binds to ARE sequences on the promoter regions of genes involved in the antioxidant defense. Active Nrf2 levels are also being regulated by autophagy and p62, a ubiquitin-binding protein. Oxidative stress upregulates p62 resulting in sequestration of Keap1, Nrf2 activation and expression of Nrf2-dependent antioxidant defense gene (Tu *et al.* 2019; Wu *et al.* 2022).

To cope with increased ROS, cells possess enzymatic and non-enzymatic antioxidant systems. One of the enzymatic antioxidants is glutathione peroxidase (GPX) that catalyzes the reduction of hydrogen peroxide and lipid peroxidase to less harmful substances like alcohol and water by using glutathione (GSH) as an electron donor. (Zhang L *et al*. 2018). The ratio of the oxidized form of glutathione (GSSG) and the reduced form (GSH) is a dynamic indicator of the oxidative stress of an organism (Jones 2002). Superoxide dismutases (SOD) convert superoxide anions to hydrogen peroxide and oxygen catalase (CAT) which was the first antioxidant enzyme to be identified catalyzes hydrogen peroxide conversion to water and oxygen. In addition, the thioredoxin systems 1 and 2 are mainly used to reduce oxidized proteins (Kurutas 2015; Kohlgrüber *et al.* 2017). SOD have three isoforms, SOD1 located in cytosol and nucleus maintains NO levels within the endothelium, SOD2 located in mitochondria maintains redox state against mitochondrial ROS, SOD3 located in extracellular matrix prevents the endothelial NO inactivation at extracellular matrix (Sah *et al.* 2020; Pourvali, Abbasi, & Mottaghi 2016). SOD was also reported to have other functions, such as RNA metabolism regulation, nuclear gene transcription activation, and glucose modulation (Eleutherio *et al.* 2020).

Peroxiredoxin (PRDX) is a ubiquitous enzyme catalyzing oxidative stress defense by rapidly neutralizing hydrogen peroxide, organic peroxides and peroxynitrite (Perkins *et al.* 2015). PRDX also affects proliferation, angiogenesis, senescence, as well as apoptosis (Perkins, Poole, & Karpus 2014).

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Materials and methods

Animals Used in the Experiment

Ethics Statement

The rabbits used in this study were New Zealand white rabbits. Wild type animals are from Innovo Ltd., Isaszeg, Hungary, the Nox4-KO animals were produced and breed at the animal facility of MATE-GBI. All animal studies were conducted in strict accordance with the recommendations and rules in the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes, approved by the Animal Welfare and Research Ethics Committee at Agricultural Biotechnology Institute, National Agricultural Research and Innovation Centre and the Hungarian University of Agriculture and Life Sciences-Institute of Genetics and Biotechnology and registered under permission number ÁK-ENG (PE/EA/58-2-2018) and PE/EA/00741-7/2022 from Pest County's governmental office. All efforts were made to minimize the suffering of animals.

Animals

Young mature adult males were used. Nox-deficient (Nox4-/-) and wild type (Nox4+/+) male rabbits (aged 20 weeks) were bred. Animals were housed in individual cages with free access to food and water. Animals were kept under a standard light–dark cycle (06.00–18.00 h) at 19 °C. Nox4−/− rabbits were generated and genotyped as described by Pinter *et al.* 2020. We used an atherogenic, high-fat feed with 0,5% w/w cholesterol to induce atherosclerosis. Age-matched Nox4+/+ and NOX4−/− male rabbits (20-week-old) were fed for 4 days with 50% normal and 50% high-fat chow diet, followed by 8 weeks of 0,5% high-fat chow diet. Male animals were used because they intend to have more stable and lower cholesterol than female animals, hormonal fluctuations do not affect the experiment.

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Sample Collection

Blood samples were collected from the animals before feeding started and at the end of the atherogenic diet period. Blood samples were collected to the Applied Biosystems™ Tempus™ Blood RNA Tube, Catalog number: 4342792 according to the manufacturers protocol.

RNA Extraction

RNA extraction was performed according to the manufacturer's instructions using Invitrogen™ Tempus™ Spin RNA Isolation Kit Catalog number: 4380204. as follows:

First, the collected blood was moved into 50 ml conical tube and 1xPBS was added to bring the total volume to 12 mL. The 12 mL of mixed sample was vortexed with maximum speed for 30 seconds to ensure the contents are properly mixed. The sample was centrifuged at 4ºC at 3000 x g (rcf) for 30 minutes. The supernatant then carefully was poured off and turned the tube in vertical position on an absorbent paper for 1-2 minutes. Afterward, 400 µl of RNA Purification Resuspension Solution was added into the conical tube and vortexed briefly to resuspend the RNA pellet. The resuspended RNA was then filtered using membrane filtration and centrifugation at 16,000 g for 30 seconds. Then, 500 µl of RNA purification wash solution 1 was added to the filter and was centrifuged again at 16,000 g for 30 seconds. The purification filter was retrieved and 500 µl of RNA purification was solution 2 was added and went on centrifugation at 16,000 g for 30 seconds. The filter then was dried by another centrifugation at 16,000 g for 30 seconds. RNA then retrieved from the filter by adding 100 µl nucleic acid purification elution buffer and centrifugation at 16,000-18,000 g for 2 minutes. Finally, approximately 90 µl of the RNA was transferred into a collection tube and the collected RNA samples were stored at -80ºC.

DNase treatment of the samples was performed on the filter with RNase-Free DNase Set (QIAGEN) Then, the total RNA was measured using Thermo ScientificTM NanodropTM.

Real Time Quantitative Polymerase Chain Reaction (qPCR)

Gene expression analyses was carried out using VitaScript™ FirstStrand cDNA Synthesis Kit and protocol, as shown in Figure 3 and Figure 4. PCR mix and reaction was carried out as

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shown in table 1. 400ng RNA was transcribed in each reaction. Specific PCR primers were designed with online tool Primer3 software: https://primer3.ut.ee/ to exon-exon boundaries of the corresponding rabbit genes.

COMPONENT	VOLUME
5X VS Reaction Buffer	4 µl
VitaScript™ Enzyme Mix	1 µl
Total RNA	1-6 µl
Nuclease-free dH_2O	to 20 µl
total volume	20 µl

Figure 3. mixture of cDNA synthesis

STEP	TEMPERATURE	TIME
cDNA Synthesis	42°C	60 minutes
Inactivation of VitaScript™	80°C	10 minutes

Figure 4. cDNA synthesis protocol

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qPCR mix	1x
Mix	10
Water	2.6
Primer forward	1.2
Primer reverse	1.2
cDNA	5
Total	20

Table 1. qPCR Mixture for one reaction

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Gene	Primer	Chromosome Location	OryCun 2.0
Nrf2 ENSOCUG00000001009	F-GCTCATCCCGTAACATGCTG R-AAGTGGCTGCTCAGAATTGC	Chromosome 7: 116,895,286-117,079,121	CM000796.1
PRDX ENSOCUG00000005951 G1SQ02_RABIT	F-GCCCTACGGAGATCATTGCT R-GTGTTGATCCAAGCCAGGTG	Chromosome 13: 121,125,072-121,135,656	CM000802.1
CAT ENSOCUG00000014198	F-ACTCGCGAACCGAAACTCTC R-AGGACATCGGGTTTCTGCG	Chromosome 1: 173,711,512-173,762,873	CM000790.1
GPX1 ENSOCUG00000013331 G1T569_RABIT	F-AGTTTGGGCATCAGGAGAACG R-GAAGAGCATGAAGTTGGGCTC	Chromosome X: 61,956,423-61,957,025	CM000811.1

Table 2. Primers sequences for qPCR

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Results

tables, relative expressions KO-WT comparison

The gene expression analysis was carried out using qPCR technique resulting in cycle threshold (Ct) value data. The average data shown in Table 3 is the average data of expression fold change with equation as follows:

$$EFC = 2^{-\Delta\Delta Ct}$$

The gene expression of total of four genes of interest (GOI) Nrf2, PRDX, CAT, GPX were analyzed by comparing Ct value to housekeeping gene (HKG)'s Ct value of the experimental groups (n=2) with two technical replicates. The HKG used as comparison in the analysis was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The relative gene expression was then statistically analyzed using student's t-test in R-studio.

Summary of the results including relative expression changes of experimental groups are shown in Table 3.

As shown in Table 3, Nox+/+ rabbits at week 0 had slightly higher expression of GPX1 and PRDX1 than Nox-/- rabbits. On the contrary, Nox-/- rabbits at week 0 had more than double the higher expression of CAT and almost 10 times higher expression of Nrf2.

At week 8, Nox-/- rabbits had higher expression of GPX1 and more than 4 times higher expression of nrf2 than Nox+/+ rabbits. Nox+/+ rabbits had almost 3 times higher expression of PRDX than Nox-/- rabbits at week 8. Nox+/+ rabbits also had more than doubled number in CAT expression than Nox-/- rabbits at week 8.

Gene expression of GPX1 were observed to decrease in Nox+/+ rabbit but to increase in Nox-/- rabbit. A significant difference was also observed in CAT gene expression, Nox-/- rabbits had higher expression in the week 0, but then Nox+/+ rabbit had higher expression in the week 8. As highlighted in Figure 5, CAT gene expression in Nox+/+ rabbits had increased significantly ($t(3) = 6.914$, p-value < 0.05) but decrease insignificantly ($t(3) = -1.0323$, p-value $= 0.3779$) in Nox-/- rabbits.

In week 0, gene expression of PRDX was higher in Nox+/+ rabbit, but then changed in week 8 resulting higher expression in Nox-/- rabbit. Significant increase of PRDX gene

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expression was observed ($t(4) = 3.7004$, p-value < 0.05) in Nox-/- rabbit while gene expression of PRDX was observed to decrease but not significant ($t(4) = -1.3612$, p-value = 0.2451) in Nox+/+ rabbits between weeks [Figure 6].

Between weeks, gene expression of Nrf2 in Nox-/- rabbit had decreased significant ($t(4) = -3.2178$, p-value = 0.0323) from 4.11 to 1.056 and a decrease but insignificant ($t(3) = -1.739$, p-value = 0.157) in Nrf2 gene expression was observed in Nox+/+ rabbit as shown in Figure 7.

There are no significant differences ($t(4) = 0.147$, p-value = 0.89; $t(4) = 0.937$, p-value = 0.402) of GPX1 expression between weeks in both of Nox+/+ and Nox-/- rabbits [Figure 8].

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Week	Gene	Rabbit	Average	Standard Deviation
W0	GPX1	KO	0.41	0.214
		WT	0.82	0.311
	NRF2	KO	3.81	2.211
		WT	0.39	0.175
	PRDX	KO	0.51	0.259
		WT	0.91	0.279
	CAT	KO	2.17	1.027
		WT	0.98	0.597
W8	GPX1	KO	0.58	0.269
		WT	0.83	0.093
	NRF2	KO	0.92	0.402
		WT	0.24	0.071
	PRDX	KO	1.24	0.584
		WT	0.42	0.147
	CAT	KO	1.67	0.359
		WT	3.59	0.757

Table 3. Relative Gene Expression of KO (Nox+/+) and WT (Nox-/-) Rabbit

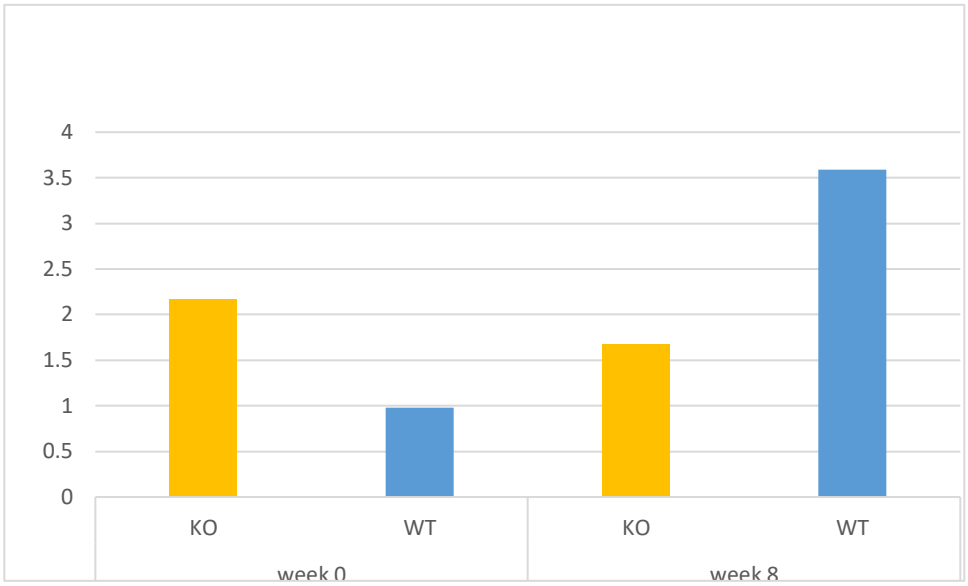

Figure 5. Relative expression of CAT Gene between experimental groups WT (Nox+/+) and KO (Nox-/-) before (week 0) and after (week 8) treatment.

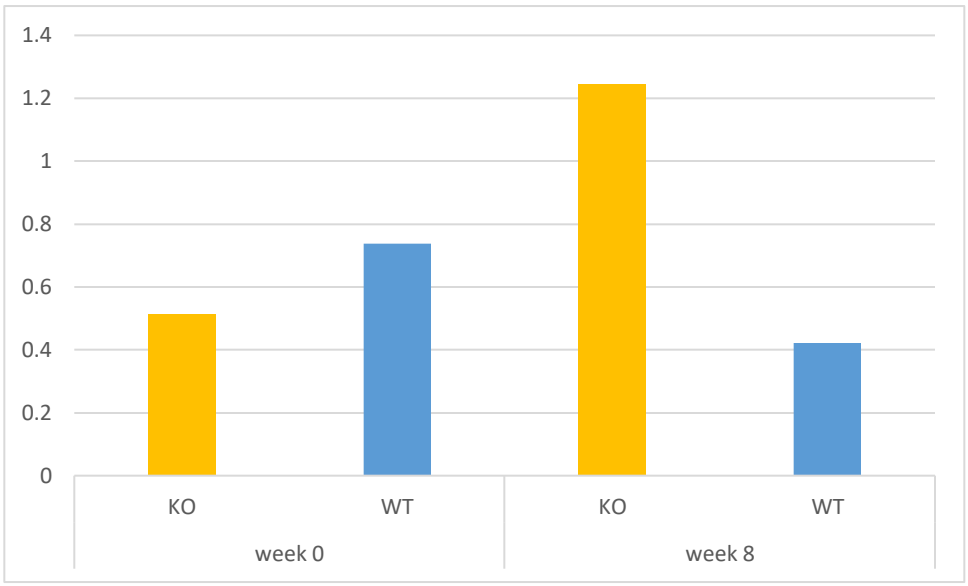

Figure 6. Relative expression of PRDX Gene between experimental groups WT (Nox+/+) and KO (Nox-/-) before (w0) and after (w8) treatment.

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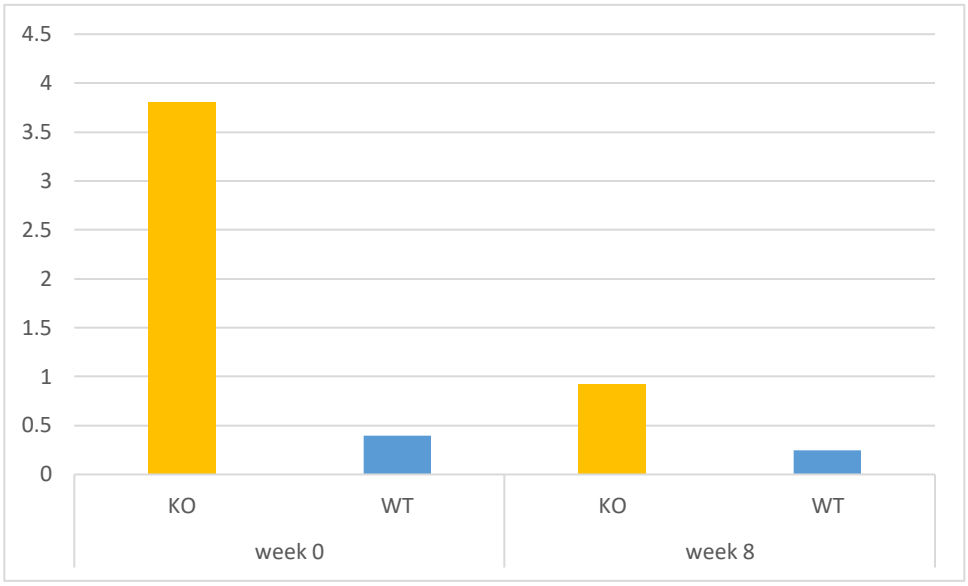

Figure 7. Relative expression of Nrf2 Gene between experimental groups WT (Nox+/+) and KO (Nox-/-) before (w0) and after (w8) treatment.

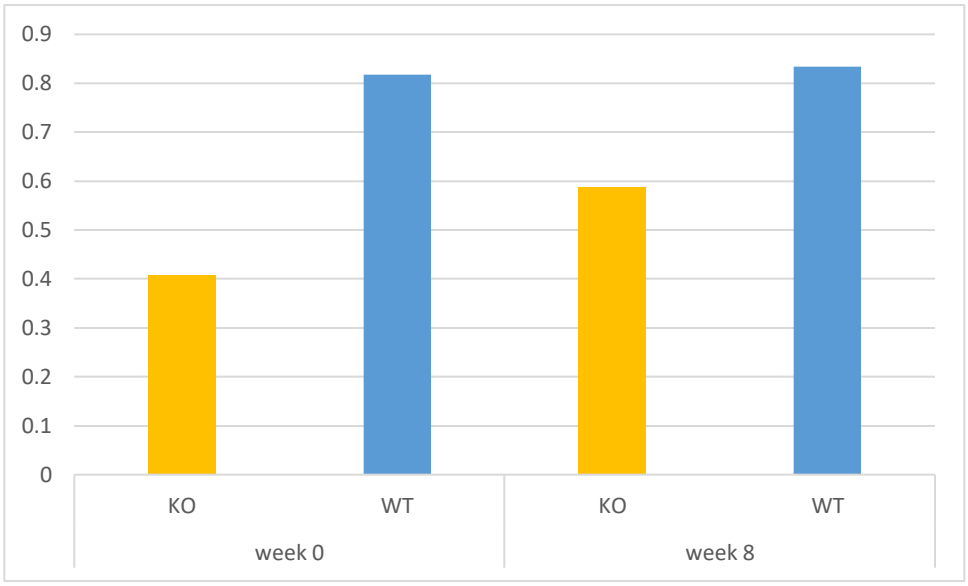

Figure 8. Relative expression of GPX1 Gene between experimental groups WT (Nox+/+) and KO (Nox-/-) before (w0) and after (w8) treatment.

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Discussion

Blood is an ideal substitution tissue for coronary artery disease (CAD) research. Blood tests may be of paramount importance in the diagnosis and prognosis of cardiovascular disease. Due to the dynamic nature of the transcriptome, genetic expression profiling has emerged as an important research tool for elucidating disease pathophysiology and finding specific biomarkers. Transcriptomic analysis of blood had shown connection to clinical symptoms related coronary angiography, myocardial infarction, plaque characteristics (Barret *et al.* 2018; McCaffrey 2021; Andreini *et al.* 2022).

Gene expression is being regulated at several phases, including transcription (which copies genetic material from genomic DNA into RNA), translation (which produces polypeptide chains based on mRNA), and post-translational modification (mRNA), post translational modification (chemical changes of protein after translation). Total RNA must be collected from the experimental sample before the mRNA can be reverse-transcribed into complementary DNA (cDNA) and utilized as the template for the qPCR reaction. This is necessary when using qPCR to measure gene expression by measuring the quantity of mRNA (Kuang *et al.* 2018).

Quantitative PCR, commonly known as real-time polymerase chain reaction (real-time PCR), is a variation of the PCR method that enables real-time monitoring of the PCR progress. In vitro amplification of a chosen DNA area across several orders of magnitude using the PCR method results in hundreds to millions of copies of a particular DNA segment. Template DNA, primers, nucleotides (dNTPs), and thermostable DNA polymerase are required components. One of the main advantages of real-time PCR over basic PCR is that this technique offers a reliable quantification relationship between the number of starting target sequences (before the amplification by PCR) and the amount of amplicon accumulated in a given PCR cycle. This relationship is provided in addition to improved accuracy, sensitivity, and speed. For the accurate quantification of the target nucleic acids, which is crucial for mRNA quantification in gene expression studies (Kubista *et al.* 2006; Artika, Wiyatno, & Ma'roef 2020). Moreover, there is no requirement for post-PCR procedures, decreasing the possibility of cross-contamination brought on by earlier amplicons. As a result, the detection and quantification of target nucleic acids has been revolutionized by this real-time PCR technology, which has also garnered a wide range of applications (Artika *et al.* 2022).

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A key factor in the variability and lack of repeatability typically seen in RT-PCR experiments is the deceptively simple process of turning RNA into a cDNA template. This is due to a number of factors. The first is that there will always be some inherent fluctuation in RNA extracted from biological samples due to the dynamic nature of cells. Second, once extracted, pure RNA might be of varying quality and is generally unstable. Thirdly, the quantity of templates affects how well RNA-to-cDNA conversion works. When target templates are uncommon, it is much lower (Bustin *et al.* 2005). Specific mistakes will be introduced into real-time quantitative PCR investigations due to minute variations in the quality or quantity of starting RNA or in the efficiency of DNA synthesis and PCR amplification. A cellular RNA is simultaneously amplified with the target in order to serve as an internal reference against which other RNA values can be normalized. The most common genes used for normalization are called housekeeping genes, such as β-actin, a cytoskeletal protein, GADPH, a glycolytic enzyme, and ribosomal RNA (Arya 2005).

Housekeeping genes are the genes that were thought to be essential, expressed at stable and constant rate and also typically related to the basic cell functions, such as cellular maintenance and evolutionary conserved. However, the stability expression of gene is affected by the cell types and condition. Thus, a reliable housekeeping gene as a comparison in translational research is essential, otherwise it would lead to false results and interpretation (Freitas *et al.* 2019; Joshi 2022).

The most popular approach for normalizing mRNA data is known as the comparative Cq or the Cq method, which involves normalization using a reference gene that is persistently expressed as an internal control. Prior to comparing values between samples, the comparative Cq approach normalizes a target gene's Cq value to internal reference genes. For each sample, the difference in the Cq values (ΔCq) of the target gene and the geometric mean of many reference genes is first computed. Then, the difference in the Cq values (ΔΔCq) between two samples is determined (e.g., control and treatment, or pre and post treatment). The calculation for the fold-change in expression between the two samples is $2^{-\Delta\Delta Cq}$, where 2 comes from 1 + efficiency and efficiency is taken to be 1 (Kuang *et al.* 2018).

In this present study, GADPH is one of the most frequently used housekeeping genes. GADPH enzyme catalyzes the glyceral-3-phospahte (G3P) into 1,3-biphospoglycerate in the

presence of NAD^+ and inorganic phosphate which is an essential reaction in the glycolytic pathway. Yet, according to recent findings, the expression levels of the widely used reference GAPDH gene varied significantly depending on the tissues or the experimental settings. Moreover, a transcriptome study of 95 human tissues from 27 distinct tissues from 95 people showed that the number of human GAPDH transcripts varied by organ and showed tissue-specific expression. However, this issue may be covered by creating PCR primers that allow for differentiation between cDNA, genomic-derived PCR, and genomic sequence information. GADPH are suitable as preference genes for study of genetic expression in rabbit and heart disease study (Li *et al.* 2017; Ma *et al.* 2015; Kwon *et al.* 2021).

NOX enzymes are responsible for causing oxidative stress by producing vascular ROS and mediating LDL peroxidation which creates ox-LDL, an inducer for inflammation and misconduct immune response in atherosclerosis (Alfarisi *et al.* 2020; Batty, Bennett, & Yu 2022). The NOX family constitutes of 7 isoforms, NOX1-5 and DUOX1-2. NOXs were characterized by large catalytic subunits which form heterodimers. P22phox and Duoxa1/2 are transmembrane proteins which respectively support the assembly and maturation of the transmembrane Nox and Duox enzymes. NOX protein activation is a complex process mediated by protein-protein interaction and controlled by several pathways. In exception, Calcium EF hands induce direct activation of NOX5 and DUOX1-2. Respectively, NOX4 has constitutive activity, mainly regulated by protein expression. In accordance with expression pattern, Nox1 is dominantly expressed in epithelial cells, Nox2 dominantly expressed in leukocytes, Nox3 limited in the inner ear, Duox enzymes are mainly expressed in the thyroid gland. While the information of Nox5 expression is still limited since the enzyme is not expressed in rodents. In contrast, Nox4 is highly expressed on the mRNA level in most of differentiated cells, and on the protein levels, the enzyme is highly detected in kidney (Brandes *et al.* 2014; Panday *et al.* 2015; Poznyak *et al.* 2020).

Nox4 is the main focus being studied in this study. The study of NOXs enzyme's function is well established which is called oxidative burst, NOX-mediated ROS release that leads to elimination of invading pathogens and mediating inflammation. However, its expression up-regulation has considered to be key pathological in numerous diseases by inducing oxidative stress (Manea 2015). Contrary to Nox1 and Nox2 which have been shown to be having proatherosclerotic role, numerous studies have shown that Nox4 has the opposite role. Nox4 has

protective activities against atherosclerosis such maintaining eNOS and heme oxygenase-1 expression, inhibiting VSMC proliferation prevents vascular inflammation and remodeling. Nox4 deletion has shown reduced superoxide and increased proinflammatory markers (Förstermann, Xia, & Li 2017; Gray *et al.* 2016). Nox4 KO mice showed enhanced media hypertrophy, atherosclerosis plaque formation, and endothelial dysfunction. While endothelial Nox4 overexpression resulted in increased vasodilation and reduced hypertension induced by angiotensin-II (Morawietsz 2018). Despite these studies, Nox4 has also shown deleterious mechanism in other diseases. Nox4 increase has been associated with stroke and myocardial infraction in late atherosclerosis (Xu *et al.* 2014). Dual inhibitor of Nox1/4 are suggested to reduce renal pathology in diabetes melitus 1 (Gorin *et al.* 2015). One of the reasons why non-specific, antioxidant therapies have failed to show any favorable outcomes in heart disease is the variety of effects of NOX4 activation in the cardiovascular system. The need for the identification of particular targets for therapeutic modulation is demonstrated by the interaction of redox pools with harmful and/or positive effects shown in Figure 9. Over these studies of Nox4, it is suggested that the enzyme appears to function in the context of atherosclerosis in a time-, cell-, and disease-specific way, and that NOX4 generally appears to perform an atheroprotective role (Gray, Shah, & Smyrnias 2019).

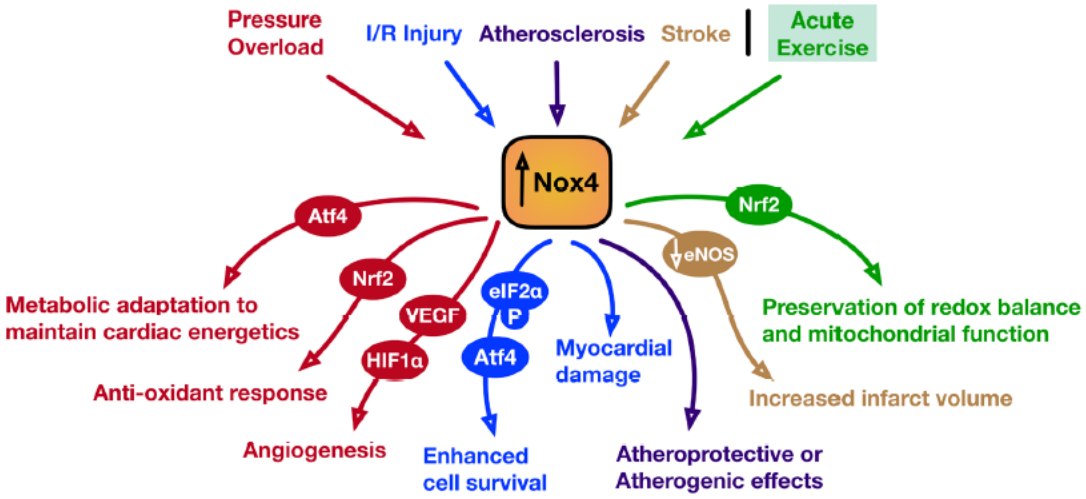

Figure 9. The pathophysiological and physiological effects of NOX4 under various conditions of cardiovascular stress

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Our result in this present study shows that NOX4 can alter the expression of antioxidant gene. While overall cholesterol rich diet can reduce the gene expression of Nrf2, the KO NOX4 rabbits showed declined expression CAT but increased expression of PRDX. Overexpression of CAT is observed to reduce vascular cells responses to oxLDL, atherosclerosis development, and F2-isoprostanes levels in the aorta of the ApoE−/− mice. In addition, SOD alone did not result in the same manner of CAT (Yang *et al.* 2004). In mice used to study the development of heart failure, EUK-8, a synthetic drug that mimics both SOD and catalase activity via selenium and manganese, prevents cardiac decompensation and remodeling of the left ventricle (van Empel *et al.* 2006). SOD activity, a key cellular superoxide defense mechanism, was only marginally elevated, but Gpx activity was marginally downregulated in compared to the control group. In comparison to the control group, the amount of CAT, which is very effective in high-level oxidative stress, was considerably higher in patients with atherosclerosis (Bogdanska *et al.* 2016). Upregulated CAT expression by pterostilbene resulted in modulating signaling activation pathway and reducing VSMC proliferation induced by ox-LDL (Wang *et al.* 2019).

Deficiency of PRDX 1/2 associated with accelerated atherogenesis and increased immune cell adhesion, endothelial expression and lesions size (Kang and Kang 2013). PRDX2 has an atheroprotective role by inhibiting the VSMC phenotype alteration and function via MAPK signaling pathway (Li *et al.* 2021). PRDX4 is localized in the endoplasmic reticulum (ER) and prevents ER stress. It has been demonstrated that overexpression of PRDX4 slows the progression of atherosclerosis by restricting the infiltration of T-cells, lowering the OS, and improving necrosis (Guo *et al.* 2012). PRDX4 was investigated to have protective role against atherosclerosis by decreasing local and systemic oxidative stressors, reducing apoptosis, and preventing migration of inflammatory cells (Yamada and Guo 2018).

Based on the results, ROS or oxidative stress results in the upregulation of antioxidant expression through the Nrf2-keap1 pathway. The activation of the Nrf2-keap1 pathway is a complex process that involves the dissociation of Nrf2 from Keap1 and its translocation to the nucleus. The Nrf2-keap1 pathway plays a crucial role in regulating the expression of various antioxidant genes, leading to an increase in antioxidant enzymes' expression (Figure 10). The Nrf2-keap1 pathway is involved in various physiological and pathological conditions such as neurodegenerative diseases and cancer, where oxidative stress plays a critical role. Overall, understanding the molecular mechanisms underlying the Nrf2-keap1 and antioxidant pathway's

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regulation provides valuable insights into the development of new therapeutic interventions to combat oxidative stress-related diseases.

However, the role of NOX4 in atherosclerosis cannot be concluded just yet, and further studies are needed to fully understand its contribution to the disease. The conflicting findings from different studies may be due to differences in experimental models, cell types, or methodologies used. Nonetheless, NOX4 remains a promising therapeutic target for atherosclerosis, and developing NOX4 inhibitors may be a potential strategy to prevent or treat the disease. Further and broader research is needed to determine the exact role of NOX4 in atherosclerosis and its potential as a therapeutic target.

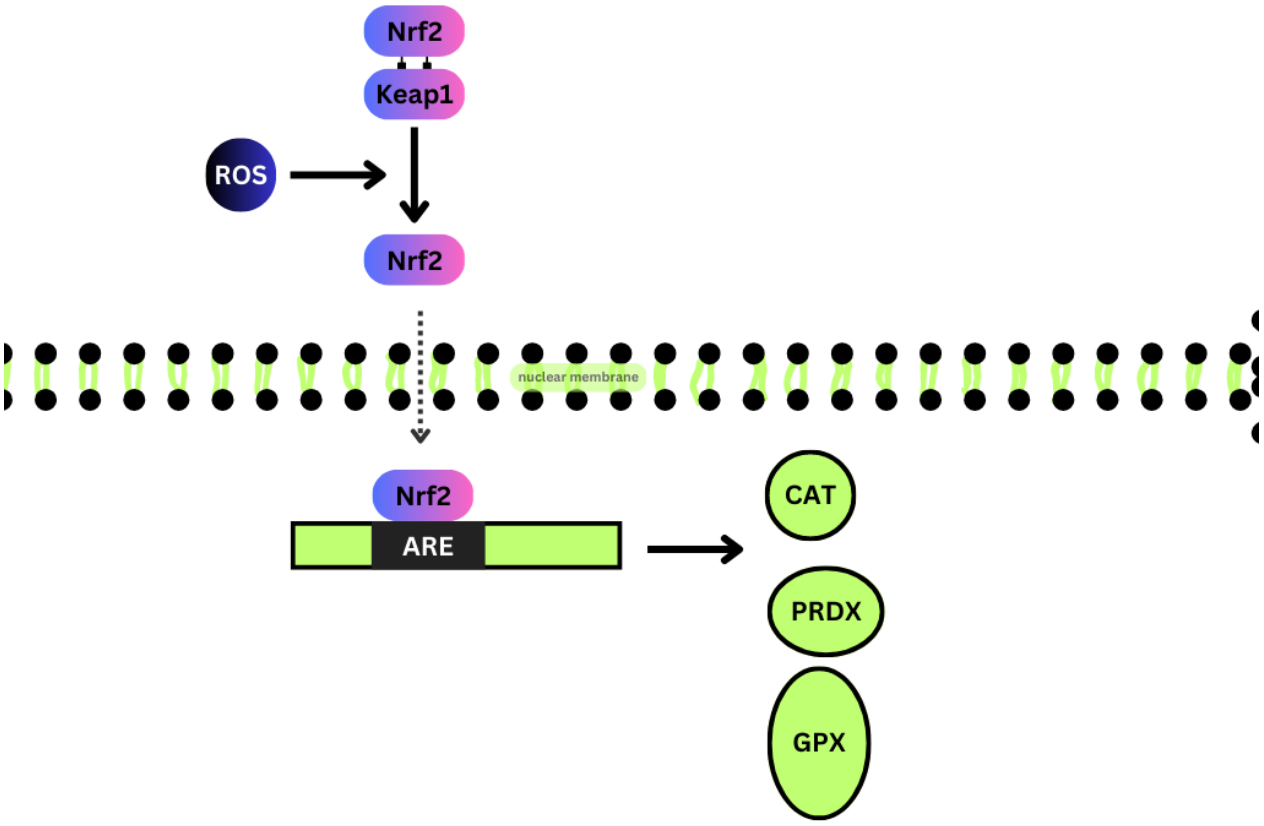

Figure 10. Schematic illustration of ROS feedback resulting in antioxidant expression through the Nrf2-Keap1 pathway

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Summary

Depending on the cell type that expresses the NOX enzyme, the levels of released chemical, and its subcellular location, hydrogen peroxide and other ROS can both operate as protective and harmful agents. Nox4 has both beneficial and deleterious role in atherosclerosis and other diseases. Total inhibition or deletion of Nox4 might accelerate and worsen the pathogenesis of atherosclerosis since the enzyme also has atheroprotective role. However, it is also related to oxidative stress which plays pivotal role in atherogenesis. It is suggested to limit the expression of Nox4 in late atherosclerosis by inhibition to reduce the risk of further development and implication of atherosclerosis and other cardiovascular diseases. Target specific Nox1-2 inhibition, Nox4 limitation, and antioxidant therapy is thought to be the best medication to treat and reduce atherosclerosis and related cardiovascular diseases.

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Bibliography

Alfarisi, H. A. H., Mohamed, Z. B. H., & Ibrahim, M. B. (2020). Basic pathogenic mechanisms of atherosclerosis. *Egyptian Journal of Basic and Applied Sciences*, *7*(1), 116-125.

Andreini, D., Melotti, E., Vavassori, C., Chiesa, M., Piacentini, L., Conte, E., ... & Colombo, G. I. (2022). Whole-Blood Transcriptional Profiles Enable Early Prediction of the Presence of Coronary Atherosclerosis and High-Risk Plaque Features at Coronary CT Angiography. *Biomedicines*, *10*(6), 1309.

Arboix, A. (2015). Cardiovascular risk factors for acute stroke: Risk profiles in the different subtypes of ischemic stroke. *World Journal of Clinical Cases: WJCC*, *3*(5), 418.

Artika, I. M., Dewi, Y. P., Nainggolan, I. M., Siregar, J. E., & Antonjaya, U. (2022). Real-Time Polymerase Chain Reaction: Current Techniques, Applications, and Role in COVID-19 Diagnosis. *Genes*, *13*(12), 2387.

Artika, I. M., Wiyatno, A., & Ma'roef, C. N. (2020). Pathogenic viruses: Molecular detection and characterization. *Infection, Genetics and Evolution*, *81*, 104215.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert review of molecular diagnostics*, *5*(2), 209-219.

Bailey, J. (2019). CRISPR-mediated gene editing: scientific and ethical issues. *Trends in biotechnology*, *37*(9), 920-921.

Barrett, T. J., Lee, A. H., Smilowitz, N. R., Hausvater, A., Fishman, G. I., Hochman, J. S., ... & Berger, J. S. (2018). Whole-Blood Transcriptome Profiling Identifies Women With Myocardial Infarction With Nonobstructive Coronary Artery Disease: Findings From the American Heart Association Go Red for Women Strategically Focused Research Network. *Circulation: Genomic and Precision Medicine*, *11*(12), e002387.

Batty, M., Bennett, M. R., & Yu, E. (2022). The role of oxidative stress in atherosclerosis. *Cells*, *11*(23), 3843.

`

Baumgartner, C., Brandl, J., Münch, G., & Ungerer, M. (2016). Rabbit models to study atherosclerosis and its complications–transgenic vascular protein expression in vivo. *Progress in Biophysics and Molecular Biology*, *121*(2), 131-141.

Boas, S. M., Joyce, K. L., & Cowell, R. M. (2021). The NRF2-dependent transcriptional regulation of antioxidant defense pathways: Relevance for cell type-specific vulnerability to neurodegeneration and therapeutic intervention. *Antioxidants*, *11*(1), 8.

Bogdanska, J., Labudovic, D., Alabakovska, S., Cekovska, S., Trajkovska, K. T., & Jovanova, S. (2016). Antioxidant enzymes in patients with atherosclerosis. *Atherosclerosis*, *252*, e71.

Boren, J., Chapman, M. J., Krauss, R. M., Packard, C. J., Bentzon, J. F., Binder, C. J., ... & Ginsberg, H. N. (2020). Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel. *European heart journal*, *41*(24), 2313-2330.

Brandes, R. P., Weissmann, N., & Schröder, K. (2014). Nox family NADPH oxidases in mechano-transduction: mechanisms and consequences. *Antioxidants & redox signaling*, *20*(6), 887-898.

Brault, J., Vigne, B., Meunier, M., Beaumel, S., Mollin, M., Park, S., & Stasia, M. J. (2020). NOX4 is the main NADPH oxidase involved in the early stages of hematopoietic differentiation from human induced pluripotent stem cells. *Free Radical Biology and Medicine*, *146*, 107-118.

Burtenshaw, D., Kitching, M., Redmond, E. M., Megson, I. L., & Cahill, P. A. (2019). Reactive oxygen species (ROS), intimal thickening, and subclinical atherosclerotic disease. *Frontiers in cardiovascular medicine*, *6*, 89.

Bustin, S. A., Benes, V., Nolan, T., & Pfaffl, M. W. (2005). Quantitative real-time RT-PCR--a perspective. *Journal of molecular endocrinology*, *34*(3), 597-601.

Buvelot, H., Jaquet, V., & Krause, K. H. (2019). Mammalian NADPH oxidases. *NADPH Oxidases: Methods and Protocols*, 17-36.

Chen, Y., Zhou, Z., & Min, W. (2018). Mitochondria, oxidative stress and innate immunity. *Frontiers in physiology*, *9*, 1487.

`

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free radical biology and medicine*, *48*(6), 749-762.

Deci, S., Lemieux, S. K., Smith-Bell, C. A., Sparks, D. L., & Schreurs, B. G. (2012). Cholesterol increases ventricular volume in a rabbit model of Alzheimer's disease. *Journal of Alzheimer's disease*, *29*(2), 283-292.

Douglas, G., & Channon, K. M. (2014). The pathogenesis of atherosclerosis. *Medicine*, *42*(9), 480-484.

Dubois-Deruy, E., Peugnet, V., Turkieh, A., & Pinet, F. (2020). Oxidative stress in cardiovascular diseases. *Antioxidants*, *9*(9), 864.

Eleutherio, E. C. A., Magalhães, R. S. S., de Araújo Brasil, A., Neto, J. R. M., & de Holanda Paranhos, L. (2021). SOD1, more than just an antioxidant. *Archives of Biochemistry and Biophysics*, *697*, 108701.

Fan, J., Kitajima, S., Watanabe, T., Xu, J., Zhang, J., Liu, E., & Chen, Y. E. (2015). Rabbit models for the study of human atherosclerosis: from pathophysiological mechanisms to translational medicine. *Pharmacology & therapeutics*, *146*, 104-119.

Förstermann, U., Xia, N., & Li, H. (2017). Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circulation research*, *120*(4), 713-735.

Freitas, F. C., Depintor, T. S., Agostini, L. T., Luna-Lucena, D., Nunes, F. M., Bitondi, M. M., ... & Lourenço, A. P. (2019). Evaluation of reference genes for gene expression analysis by real-time quantitative PCR (qPCR) in three stingless bee species (Hymenoptera: Apidae: Meliponini). *Scientific Reports*, *9*(1), 1-13.

Gorin, Y., Cavaglieri, R. C., Khazim, K., Lee, D. Y., Bruno, F., Thakur, S., ... & Abboud, H. E. (2015). Targeting NADPH oxidase with a novel dual Nox1/Nox4 inhibitor attenuates renal pathology in type 1 diabetes. *American Journal of Physiology-Renal Physiology*, *308*(11), F1276-F1287.

Gray, S. P., Di Marco, E., Kennedy, K., Chew, P., Okabe, J., El-Osta, A., ... & Jandeleit-Dahm, K. A. (2016). Reactive oxygen species can provide atheroprotection via NOX4-dependent inhibition

of inflammation and vascular remodeling. *Arteriosclerosis, thrombosis, and vascular biology*, *36*(2), 295-307.

Gray, S. P., Shah, A. M., & Smyrnias, I. (2019). NADPH oxidase 4 and its role in the cardiovascular system. *Vascular Biology*, *1*(1), H59.

Guo, X., Yamada, S., Tanimoto, A., Ding, Y., Wang, K. Y., Shimajiri, S., ... & Sasaguri, Y. (2012). Overexpression of peroxiredoxin 4 attenuates atherosclerosis in apolipoprotein E knockout mice. *Antioxidants & redox signaling*, *17*(10), 1362-1375.

Helfinger, V., Palfi, K., Weigert, A., & Schröder, K. (2019). The NADPH oxidase Nox4 controls macrophage polarization in an NFκB-dependent manner. *Oxidative Medicine and Cellular Longevity*, *2019*.

Hiebert, P. (2021). The Nrf2 transcription factor: a multifaceted regulator of the extracellular matrix. *Matrix Biology Plus*, *10*, 100057.

Huang, Y., Li, W., Su, Z. Y., & Kong, A. N. T. (2015). The complexity of the Nrf2 pathway: beyond the antioxidant response. *The Journal of nutritional biochemistry*, *26*(12), 1401-1413.

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria journal of medicine*, *54*(4), 287-293.

Jebari-Benslaiman, S., Galicia-García, U., Larrea-Sebal, A., Olaetxea, J. R., Alloza, I., Vandenbroeck, K., ... & Martín, C. (2022). Pathophysiology of atherosclerosis. *International Journal of Molecular Sciences*, *23*(6), 3346.

Jiang, H., Zhou, Y., Nabavi, S. M., Sahebkar, A., Little, P. J., Xu, S., ... & Ge, J. (2022). Mechanisms of oxidized LDL-mediated endothelial dysfunction and its consequences for the development of atherosclerosis. *Frontiers in Cardiovascular Medicine*, 1442.

Jones, D. P. (2002). [11] Redox potential of GSH/GSSG couple: assay and biological significance. In *Methods in enzymology* (Vol. 348, pp. 93-112). Academic Press.

Joshi, C. J., Ke, W., Drangowska-Way, A., O'Rourke, E. J., & Lewis, N. E. (2022). What are housekeeping genes?. *PLoS computational biology*, *18*(7), e1010295.

`

Kang, D. H., & Kang, S. W. (2013). Targeting cellular antioxidant enzymes for treating atherosclerotic vascular disease. *Biomolecules & Therapeutics*, *21*(2), 89.

Kattoor, A. J., Pothineni, N. V. K., Palagiri, D., & Mehta, J. L. (2017). Oxidative stress in atherosclerosis. *Current atherosclerosis reports*, *19*, 1-11.

Keane, O. (2019). Genetically Engineered Nonhuman Animals: A Global Overview and Research Agenda. *society & animals*, *29*(2), 173-202.

Kim, J., Yoo, J. Y., Suh, J. M., Park, S., Kang, D., Jo, H., & Bae, Y. S. (2019). The flagellin-TLR5-Nox4 axis promotes the migration of smooth muscle cells in atherosclerosis. *Experimental & molecular medicine*, *51*(7), 1-13.

Kohlgrüber, S., Upadhye, A., Dyballa-Rukes, N., McNamara, C. A., & Altschmied, J. (2017). Regulation of transcription factors by reactive oxygen species and nitric oxide in vascular physiology and pathology. *Antioxidants & Redox Signaling*, *26*(13), 679-699.

Kuang, J., Yan, X., Genders, A. J., Granata, C., & Bishop, D. J. (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PloS one*, *13*(5), e0196438.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., ... & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular aspects of medicine*, *27*(2-3), 95-125.

Kurutas, E. B. (2015). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition journal*, *15*(1), 1-22.

Kwon, N., Lee, K. E., Singh, M., & Kang, S. G. (2021). Suitable primers for GAPDH reference gene amplification in quantitative RT-PCR analysis of human gene expression. *Gene Reports*, *24*, 101272.

Li, J., Wang, C., Wang, W., Liu, L., Zhang, Q., Zhang, J., ... & Sun, L. (2021). PRDX2 Protects against atherosclerosis by regulating the phenotype and function of the vascular smooth muscle cell. *Frontiers in Cardiovascular Medicine*, *8*, 624796.

Li, M., Rao, M., Chen, K., Zhou, J., & Song, J. (2017). Selection of reference genes for gene expression studies in heart failure for left and right ventricles. *Gene*, *620*, 30-35.

`

Lingappan, K. (2018). NF-κB in oxidative stress. *Current opinion in toxicology*, *7*, 81-86.

Lu, S., Liao, Z., Lu, X., Katschinski, D. M., Mercola, M., Chen, J., ... & Bers, D. M. (2020). Hyperglycemia acutely increases cytosolic reactive oxygen species via O-linked GlcNAcylation and CaMKII activation in mouse ventricular myocytes. *Circulation research*, *126*(10), e80-e96.

Ma, H., Yang, Q., Li, D., & Liu, J. (2015). Validation of suitable reference genes for quantitative polymerase chain reaction analysis in rabbit bone marrow mesenchymal stem cell differentiation. *Molecular medicine reports*, *12*(2), 2961-2968.

Manea, S. A., Constantin, A., Manda, G., Sasson, S., & Manea, A. (2015). Regulation of Nox enzymes expression in vascular pathophysiology: focusing on transcription factors and epigenetic mechanisms. *Redox biology*, *5*, 358-366.

Markin, A. M., Sobenin, I. A., Grechko, A. V., Zhang, D., & Orekhov, A. N. (2020). Cellular mechanisms of human atherogenesis: focus on chronification of inflammation and mitochondrial mutations. *Frontiers in pharmacology*, *11*, 642.

McCaffrey, T. A., Toma, I., Yang, Z., Katz, R., Reiner, J., Mazhari, R., ... & Laurent, G. S. (2021). RNA sequencing of blood in coronary artery disease: involvement of regulatory T cell imbalance. *BMC Medical Genomics*, *14*(1), 1-20.

Milkovic, L., Cipak Gasparovic, A., Cindric, M., Mouthuy, P. A., & Zarkovic, N. (2019). Short overview of ROS as cell function regulators and their implications in therapy concepts. *Cells*, *8*(8), 793.

Morawietz, H. (2018). Cardiovascular protection by Nox4. *Cardiovascular research*, *114*(3), 353-355.

Mundi, S., Massaro, M., Scoditti, E., Carluccio, M. A., Van Hinsbergh, V. W., Iruela-Arispe, M. L., & De Caterina, R. (2018). Endothelial permeability, LDL deposition, and cardiovascular risk factors—a review. *Cardiovascular research*, *114*(1), 35-52.

Nawaz, B., Fromm, A., Øygarden, H., Eide, G. E., Saeed, S., Meijer, R., ... & Waje-Andreassen, U. (2021). Prevalence of atherosclerosis and association with 5-year outcome: The Norwegian Stroke in the Young Study. *European Stroke Journal*, *6*(4), 374-384.

`

Panday, A., Sahoo, M. K., Osorio, D., & Batra, S. (2015). NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cellular & molecular immunology*, *12*(1), 5-23.

Peluso, I., Morabito, G., Urban, L., Ioannone, F., & Serafi, M. (2012). Oxidative stress in atherosclerosis development: the central role of LDL and oxidative burst. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*, *12*(4), 351-360.

Perkins, A., Nelson, K. J., Parsonage, D., Poole, L. B., & Karplus, P. A. (2015). Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. *Trends in biochemical sciences*, *40*(8), 435-445.

Perkins, A., Poole, L. B., & Karplus, P. A. (2014). Tuning of peroxiredoxin catalysis for various physiological roles. *Biochemistry*, *53*(49), 7693-7705.

Pethető, G. L., Kerekes, A., Mihálffy, M., Donkó, Á., Bodrogi, L., Skoda, G., ... & Geiszt, M. (2021). Disruption of the NOX5 gene aggravates atherosclerosis in rabbits. *Circulation Research*, *128*(9), 1320-1322.

Pintér, T., Geiszt, M., Pethető, G. L., Mihálffy, M., Skoda, G., Lipták, N., ... & Bodrogi, L. (2020). The creation of a multiallele knockout genotype in rabbit using crispr/cas9 and its application in translational medicine. *Applied Sciences*, *10*(23), 8508.

Pourvali, K., Abbasi, M., & Mottaghi, A. (2016). Role of Superoxide Dismutase 2 Gene Ala16Val Polymorphism and Total Antioxidant Capacity in Diabetes and its Complications. *Avicenna journal of medical biotechnology*, *8*(2), 48–56.

Poznyak, A. V., Grechko, A. V., Orekhova, V. A., Khotina, V., Ivanova, E. A., & Orekhov, A. N. (2020). NADPH oxidases and their role in atherosclerosis. *Biomedicines*, *8*(7), 206.

PRDX2 Protects Against Atherosclerosis by Regulating the Phenotype and Function of the Vascular Smooth Muscle Cell

Rafieian-Kopaei, M., Setorki, M., Doudi, M., Baradaran, A., & Nasri, H. (2014). Atherosclerosis: process, indicators, risk factors and new hopes. *International journal of preventive medicine*, *5*(8), 927.

`

Rastogi, R., Geng, X., Li, F., & Ding, Y. (2017). NOX activation by subunit interaction and underlying mechanisms in disease. *Frontiers in cellular neuroscience*, *10*, 301.

Roth, G. A., Mensah, G. A., Johnson, C. O., Addolorato, G., Ammirati, E., Baddour, L. M., ... & GBD-NHLBI-JACC Global Burden of Cardiovascular Diseases Writing Group. (2020). Global burden of cardiovascular diseases and risk factors, 1990–2019: update from the GBD 2019 study. *Journal of the American College of Cardiology*, *76*(25), 2982-3021.

Sah, S. K., Agrahari, G., & Kim, T. Y. (2020). Insights into superoxide dismutase 3 in regulating biological and functional properties of mesenchymal stem cells. *Cell & Bioscience*, *10*(1), 1-12.

Saha, S. K., Lee, S. B., Won, J., Choi, H. Y., Kim, K., Yang, G. M., ... & Cho, S. G. (2017). Correlation between oxidative stress, nutrition, and cancer initiation. *International journal of molecular sciences*, *18*(7), 1544.

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Current biology*, *24*(10), R453-R462.

Sharifi-Rad, M., Anil Kumar, N. V., Zucca, P., Varoni, E. M., Dini, L., Panzarini, E., ... & Sharifi-Rad, J. (2020). Lifestyle, oxidative stress, and antioxidants: back and forth in the pathophysiology of chronic diseases. *Frontiers in physiology*, *11*, 694.

Shi, Q., & Gibson, G. E. (2007). Oxidative stress and transcriptional regulation in Alzheimer's disease. *Alzheimer disease and associated disorders*, *21*(4), 276.

Sinenko, S. A., Starkova, T. Y., Kuzmin, A. A., & Tomilin, A. N. (2021). Physiological signaling functions of reactive oxygen species in stem cells: from flies to man. *Frontiers in cell and developmental biology*, *9*, 714370.

Singh, R. B., Mengi, S. A., Xu, Y. J., Arneja, A. S., & Dhalla, N. S. (2002). Pathogenesis of atherosclerosis: A multifactorial process. *Experimental & Clinical Cardiology*, *7*(1), 40.

Skonieczna, M., Hejmo, T., Poterala-Hejmo, A., Cieslar-Pobuda, A., & Buldak, R. J. (2017). NADPH oxidases: insights into selected functions and mechanisms of action in cancer and stem cells. *Oxidative Medicine and Cellular Longevity*, *2017*.

`

Tarafdar, A., & Pula, G. (2018). The role of NADPH oxidases and oxidative stress in neurodegenerative disorders. *International Journal of Molecular Sciences*, *19*(12), 3824.

Rastogi, R., Geng, X., Li, F., & Ding, Y. (2017). NOX activation by subunit interaction and underlying mechanisms in disease. *Frontiers in cellular neuroscience*, *10*, 301.

Teshima, Y., Takahashi, N., Nishio, S., Saito, S., Kondo, H., Fukui, A., ... & Saikawa, T. (2014). Production of Reactive Oxygen Species in the Diabetic Heart–Roles of Mitochondria and NADPH Oxidase–. *Circulation Journal*, *78*(2), 300-306.

Tu, W., Wang, H., Li, S., Liu, Q., & Sha, H. (2019). The anti-inflammatory and anti-oxidant mechanisms of the Keap1/Nrf2/ARE signaling pathway in chronic diseases. *Aging and disease*, *10*(3), 637.

Vaduganathan, M., Mensah, G. A., Turco, J. V., Fuster, V., & Roth, G. A. (2022). The Global Burden of Cardiovascular Diseases and Risk: A Compass for Future Health. *Journal of the American College of Cardiology*, *80*(25), 2361-2371.

van Empel, V. P., Bertrand, A. T., van Oort, R. J., van der Nagel, R., Engelen, M., van Rijen, H. V., ... & De Windt, L. J. (2006). EUK-8, a superoxide dismutase and catalase mimetic, reduces cardiac oxidative stress and ameliorates pressure overload-induced heart failure in the harlequin mouse mutant. *Journal of the American College of Cardiology*, *48*(4), 824-832.

Vodjgani, M., Salehi, Z., & Izad, M. (2020). The influence of reactive oxygen species in the immune system and pathogenesis of multiple sclerosis. *Autoimmune diseases*, *2020*.

Wang, M., Liu, Y., Liang, Y., Naruse, K., & Takahashi, K. (2021). Systematic understanding of pathophysiological mechanisms of oxidative stress-related conditions—diabetes mellitus, cardiovascular diseases, and ischemia–reperfusion injury. *Frontiers in cardiovascular medicine*, *8*, 649785.

Wang, W., Wang, Y. R., Chen, J., Chen, Y. J., Wang, Z. X., Geng, M., ... & Sun, J. (2019). Pterostilbene attenuates experimental atherosclerosis through restoring catalase-mediated redox balance in vascular smooth muscle cells. *Journal of agricultural and food chemistry*, *67*(46), 12752-12760.

`

Wu, X., Wei, J., Yi, Y., Gong, Q., & Gao, J. (2022). Activation of Nrf2 signaling: A key molecular mechanism of protection against cardiovascular diseases by natural products. *Frontiers in Pharmacology*, *13*.

Xu, S., Chamseddine, A. H., Carrell, S., & Miller Jr, F. J. (2014). Nox4 NADPH oxidase contributes to smooth muscle cell phenotypes associated with unstable atherosclerotic plaques. *Redox biology*, *2*, 642-650.

Yaghi, S., Prabhakaran, S., Khatri, P., & Liebeskind, D. S. (2019). Intracranial atherosclerotic disease: mechanisms and therapeutic implications. *Stroke*, *50*(5), 1286-1293.

Yamada, S., & Guo, X. (2018). Peroxiredoxin 4 (PRDX4): Its critical in vivo roles in animal models of metabolic syndrome ranging from atherosclerosis to nonalcoholic fatty liver disease. *Pathology International*, *68*(2), 91-101.

Yang, H., Roberts, L. J., Shi, M. J., Zhou, L. C., Ballard, B. R., Richardson, A., & Guo, Z. M. (2004). Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. *Circulation research*, *95*(11), 1075-1081.

Yang, X., Li, Y., Li, Y., Ren, X., Zhang, X., Hu, D., ... & Shang, H. (2017). Oxidative stress-mediated atherosclerosis: mechanisms and therapies. *Frontiers in physiology*, *8*, 600.

Yu, C., & Xiao, J. H. (2021). The Keap1-Nrf2 system: a mediator between oxidative stress and aging. *Oxidative Medicine and Cellular Longevity*, *2021*, 1-16.

Zhang, L., Wu, M., Yu, D., Teng, Y., Wei, T., Chen, C., & Song, W. (2018). Identification of glutathione peroxidase (GPX) gene family in rhodiola crenulata and gene expression analysis under stress conditions. *International Journal of Molecular Sciences*, *19*(11), 3329.

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiological reviews*, *94*(3), 909-950.

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