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Effect of redox signaling molecules on NRF2 pathway

Summary of studies conducted by:

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Introduction

Nrf2 – a transcription factor

Erythroid-specific nuclear factor 2 (NF-E2) was first characterized, described, and cloned in murine by Andrews et al, in 1993 This polypeptide was a new hematopoietic cell-specific member of the basic region-leucine zipper family (b-zip) of transcription factors (1). NF-E2 is one of the proteins that bind to the tandem AP1 (activating protein 1)-NF-E2 repeat, which represents the core enhancer within HS2 (2).

In 1994 it was discovered a member of the human CNC-bZIP transcription factor family for the transcriptional stimulation of beta-globin genes, the Nrf2 or NF-E2-related factor 2 (previously HEBP1) as previously known. It was cloned, its genomic structure isolated and named for the first time as Nuclear Factor Erythroid 2-Related Factor, or Nrf2. It was described as a Nf-E2-like basic leucine zipper transcriptional activator. Interesting to observe is that in contrast to NF-E2, the expression of Nrf2 is not restricted to the hematopoietic tissues. At that time, it was not known what role Nrf2 played in nonhematopoietic tissues where it was expressed at high levels. The high degree of conservation between the mouse and human Nrf2 genes suggested that they, intriguingly, have an important function (2). Nrf2 is encoded by the nuclear factor (erythroid-derived 2)-like 2 gene (NFE2L2) located in the cytogenetic band 2q31.2 of chromosome 2 in humans (gene ID: 4780). The NFE2L2 consists of five exons interrupted by four introns giving a rise of 8 transcript variants encoding 6 isoforms of Nrf2 (3).

Decades later, much more is known about this protein. According to Cancer.gov/National Institute of Health, Nrf2 is a protein that controls how certain genes are expressed. These genes help to protect cells from damage caused by free radicals (reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems, 4). These free

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radicals are important compounds because they may play an important part in the development of certain conditions, and they are also related to the aging process (5). Current evidence has shown that NRF2 lies at the center of a complex regulatory network and establishes Nrf2 as a truly pleiotropic transcription factor (6).

Nrf2 is a master regulator of the antioxidant response and xenobiotic metabolism through the regulation of a wide range of antioxidant and phase II detoxification genes. Consequently, Nrf2 protects cells from different types of stressors, from endogenous substances and reactive oxygen species (ROS) to environmental insults (7).

Nrf2 activity is regulated by Keap1 (Kelch-like ECH-associated protein 1) – an adaptor subunit of Cullin 3-based E3 ubiquitin ligase and acts as a sensor for oxidative and electrophilic stresses (8). Keap1 acts as a key sensor of oxidative and electrophilic stress. Under homeostatic conditions, KEAP1 forms part of an E3 ubiquitin ligase, which tightly regulates the activity of the transcription factor NRF2 by targeting it for ubiquitination and proteasome-dependent degradation. In response to stress, an intricate molecular mechanism facilitated by sensor cysteines within KEAP1 allows NRF2 to escape ubiquitination, accumulate within the cell, and translocate to the nucleus, where it can promote its antioxidant transcription program, regulating cytoprotective gene expression (9).

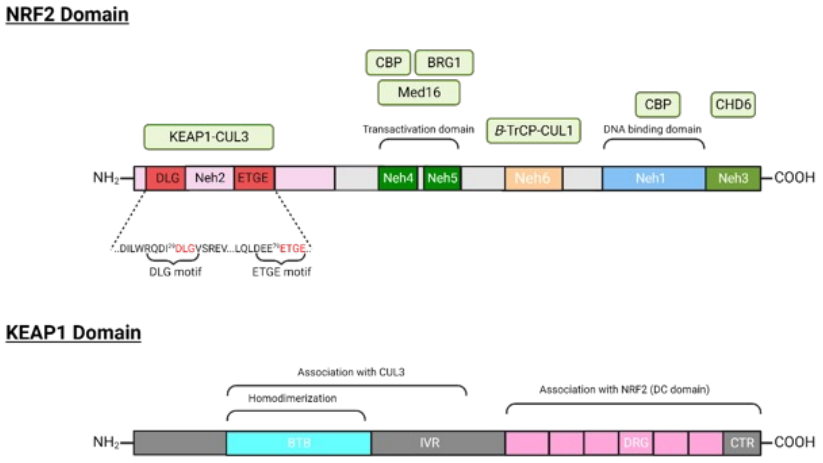

Figure 1:

Domain structures of NRF2 (A) and KEAP1 (B). NRF2-interacting molecules are shown in green boxes and placed above their interacting domains (Adapted and modified from reference 10).

Phylogenetic conservation of the structure of NRF2 among species revealed the presence of six functional domains: Neh1 (NRF2-ECH homology domain-1) to Neh6. Neh1 contains the CNC and bZIP domains that mediate DNA binding and dimer formation, whereas Neh3, Neh4, and Neh5 are transactivation domains. Of the transactivation domains, Neh4 and Neh5 make a major contribution to transcriptional activation by recruiting histone acetyl-transferase cAMP responsive element binding protein and Mediator Complex (9).

KEAP1 acts as a cysteine thiol-rich sensor of redox insults, whereas NRF2 is a transcription factor that robustly transduces chemical signals to regulate a battery of cytoprotective genes. KEAP1 represses NRF2 activity under quiescent conditions, whereas NRF2 is liberated from KEAP1-mediated repression on exposure to stresses. The KEAP1-NRF2 system is an effective apparatus

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for maintaining redox homeostasis and the defense mechanism against environmental insults (10).

Intensive research on the KEAP1-NRF2 system demonstrated the critical significance of NRF2 activity and its regulatory mechanisms to the maintenance of our health. Important to highlight that dysregulation of this system underlies the pathogenesis of various human diseases (11).

The most important feature of NRF2 is its inducibility. KEAP1 creates the inducible nature of NRF2 function by serving as a substrate recognition component of the E3 ubiquitin ligase complex in cooperation with CUL3 and RBX. Under normal conditions, ubiquitinated NRF2 undergoes proteasomal degradation, and it is this constitutive degradation of NRF2 that maintains the quantity and activity of NRF2 at a low level. The E3 ubiquitin ligase activity of the KEAP1-CUL3 complex is disrupted on exposure to electrophiles and ROS that modify the cysteine residues of KEAP1, allowing newly synthesized NRF2 to accumulate in the nucleus and activate transcription. Thus, the possession of highly reactive cysteine residues renders KEAP1 an efficient and sensitive biosensor of redox disturbance through switching on or off the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex (10).

Upon exposure of cells to oxidative stress or chemo preventive compounds, Nrf2 translocates to the nucleus, forms a heterodimer with its obligatory partner Maf, and binds to the ARE sequence to activate transcription of several different types of genes. The majority of the downstream genes of Nrf2 contain an ARE sequence in the promoter (12). One of the most important characteristics of Nrf2-mediated transcription is the inducibility in response to xenobiotic and

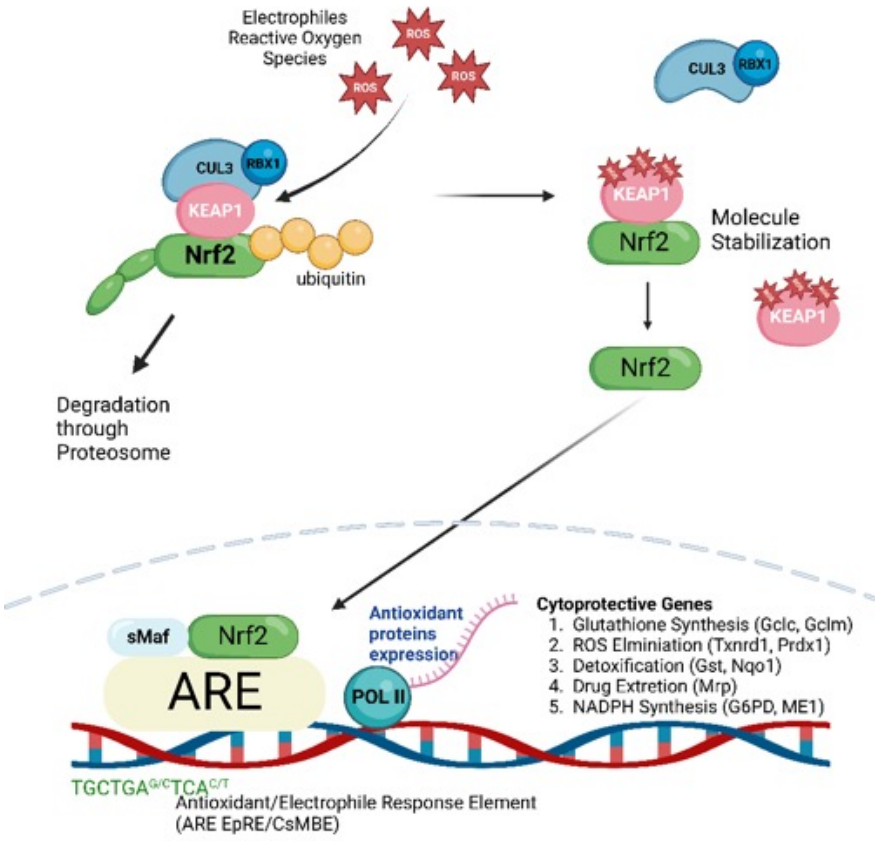

Figure 2:

KEAP1-NRF2 is a two-component system. In the cytoplasm, NRF2 is ubiquitinated by the KEAP1-CUL3 ubiquitin E3 ligase complex to mark it for degradation by the proteasome. When cells are exposed to electrophiles or reactive oxygen species, KEAP1 is modified and the KEAP1-CUL3 ubiquitin E3 ligase activity declines, which results in the stabilization of NRF2. Stabilized and accumulated NRF2 translocates to the nucleus and activates a battery of cytoprotective genes (Adapted and modified from reference 10).

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oxidative stresses. Under normal conditions, the activity of the Nrf2-mediated transcription is low, as most of Nrf2 protein is degraded in the proteasome (Itoh et al., 2003). When cells are exposed to electrophiles or ROS, Nrf2 is stabilized and accumulates in the nucleus, which results in the robust activation of Nrf2 target genes. Thus, the mechanism underlying Nrf2 degradation under normal conditions and the stabilization of Nrf2 following exposure to stress are critical clues to the revelation of the molecular basis of our defense system (13).

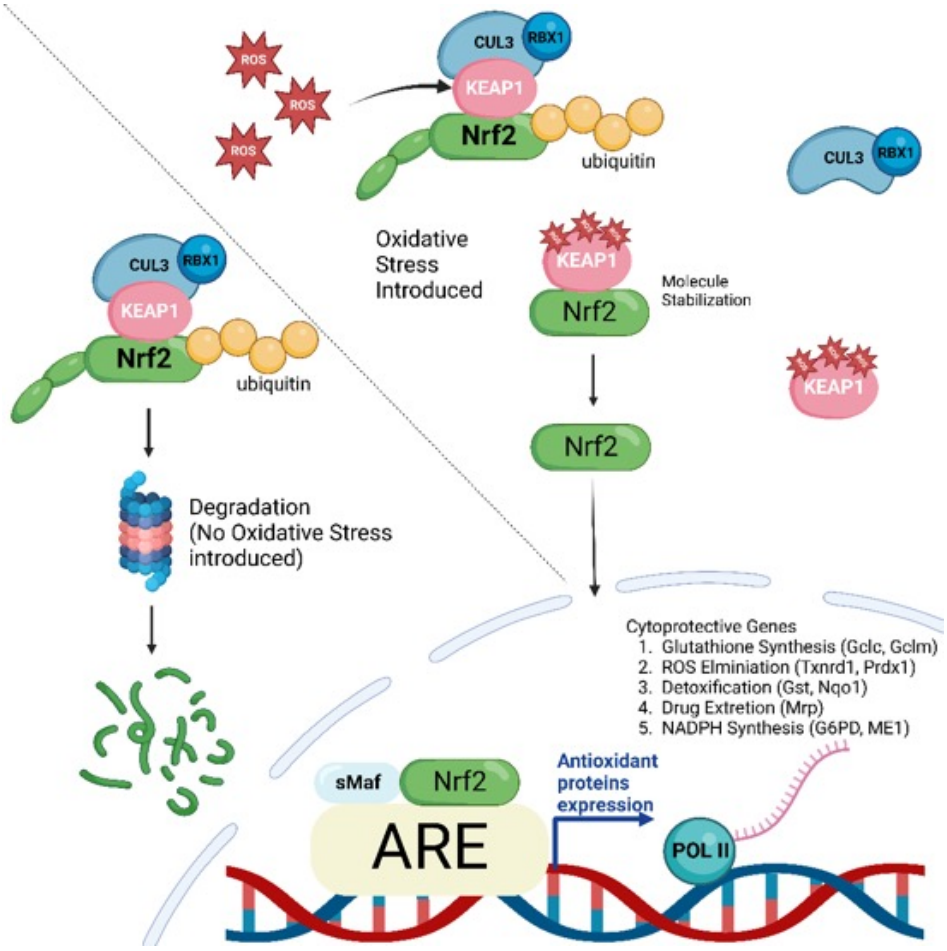

Figure 3:

The KEAP1–NRF2 System. Under normal conditions, Nrf2 is constantly ubiquitinated through Keap1 and degraded in the proteasome. Following exposure to electrophiles or oxidative stress, Keap1 is inactivated. Stabilized Nrf2 accumulates in the nucleus and activates many cytoprotective genes (Adapted and modified from reference 13).

At the end of the story, KEAP1-Nrf2 system is and effective apparatus for maintaining redox homeostasis. It is a perfect combination where KEAP1 acts as a sensor of redox insults and Nrf2 acts as a transcription factor that robustly transduces chemical signals to regulate a battery of cytoprotective genes. The relation between them resumes in such a way that KEAP1 represses NRF2 activity under quiescent conditions, whereas NRF2 is liberated from KEAP1-mediated repression on exposure to stresses (10).

Nrf2-KEAP1 pathway and regulation

The Nrf2-KEAP1 pathway is fundamental to sense and respond to oxidative and electrophilic stress. In the classical Nrf2-KEAP1 signaling pathway

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Keap1 is a key Nrf2 repressor and plays a pivotal role in regulating the Nrf2 signaling pathway. Keap1 serves as a bridge between Nrf2 and ubiquitination ligase Cullin-3. Oxidative stressors or electrophiles inhibit the ubiquitination-dependent degradation and increase nuclear accumulation of Nrf2. As a cysteine-rich protein, Keap1 is an excellent sensor for chemical inducers (14).

Nrf2 is a transcription factor affecting the expression of many different genes involved in antioxidant stress and drug metabolism. Thus, regulation of Nrf2 expression is an important target for treating or preventing disease and this regulation has been reported to be influenced by epigenetics (15).

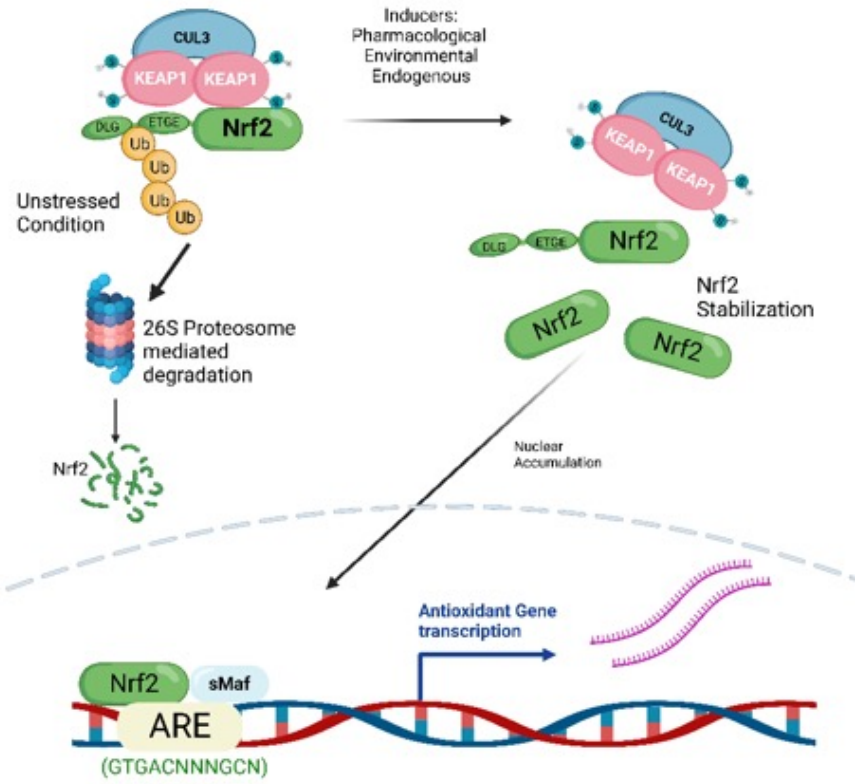

Figure 4:

Nrf2 signaling pathway schema diagram (Adapted and modified from reference 16)

Expression of Nrf2 is inherent to cells, thus ensuring protective response to oxidative stress and inflammation. Under physiological conditions, Nrf2 has a rapid turnover as a result of the ubiquitin proteasome system. The Nrf2 stability is controlled by KEAP-1, known as a redox sensor for endogenous and environmental oxidative signals as well as for electrophilic reactions (17). Nrf2 can also be regulated at the transcriptional level. The NFE2L2 gene promoter presents several regulatory sequences (18). Interesting to say that the so-called Nrf2 activators should be called "KEAP1" inhibitors. Under normal circumstances, Keap1 exists as dimers inside the cells, functioning as a substrate linker protein for the interaction of Cul3/Rbx1-based E3-ubiquitin ligase complex with Nrf2 which leads to the continuous ubiquitination of Nrf2 and its subsequent proteasomal degradation. This would maintain low basal levels of Nrf2-regulated antioxidants. Under

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cellular stress or exposure to mild oxidative stress, Nrf2 dissociates from Keap1, becomes stabilized, translocates into the nuclei where it interacts with other protein factors and binds to ARE, leading to increased transcription of antioxidant genes (10). In mammals, including humans, the Keap1-Cul3-Rbx1 axis is known as the most critical regulatory mechanism of Nrf2 activity (19).

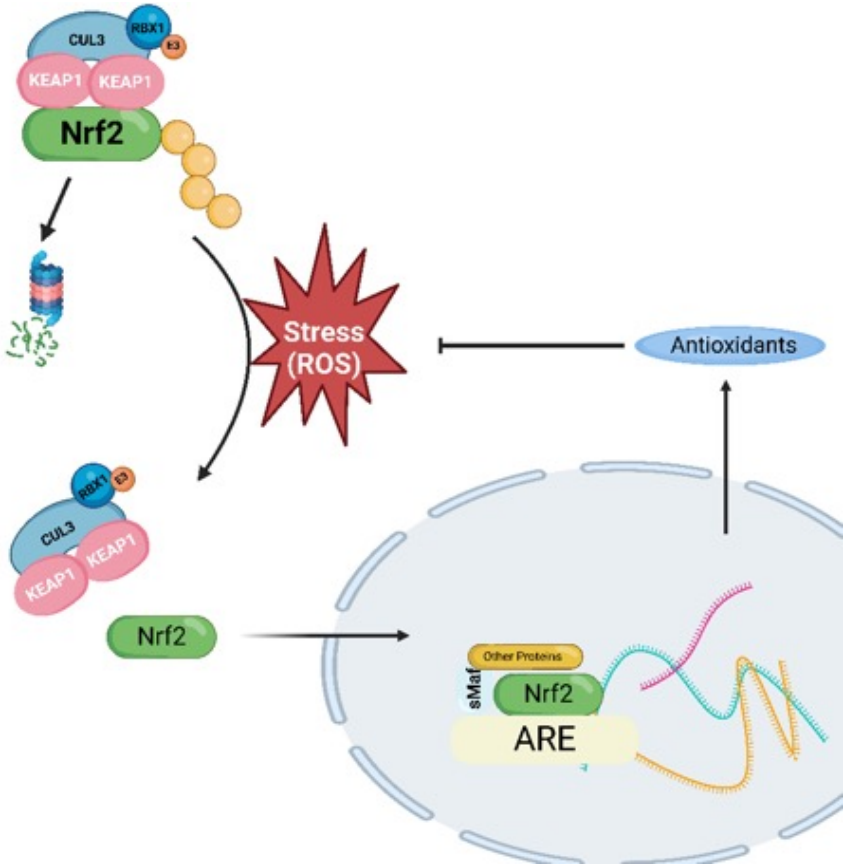

Figure 5:

Keap1 as the chief regulator of Nrf2 activation and consequent ARE-driven antioxidant gene expression (Adapted and modified from reference 19).

The predominant mechanisms by which stress conditions (e.g., oxidative and electrophilic stress) and chemical inducers cause the dissociation of Nrf2 from Keap1 is the oxidation of cysteine residues of Keap1. The cysteine sulfhydryl groups may be oxidized to form disulfide bridge, sulfenic acid, or sulfonic acid. These redox modifications cause the separation of Keap1 from Nrf2, leading to Nrf2 stabilization and nuclear translocation (19).

Deregulation of Nrf2 transcriptional activity has been described in the pathogenesis of multiple diseases, and the Nrf2/Keap1 axis has emerged as a crucial modulator of cellular homeostasis. Whereas the significance of Nrf2 in the modulation of biological processes has been well established and broadly discussed in detail, the focus on Keap1 rarely goes beyond the regulation of Nrf2 activity and redox sensing (20).

It is important to consider that the human diet provides a large set of active compounds that can affect and activate Nrf2 signaling pathway (52). Amongst them, some are even considered potent Nrf2 activators. These

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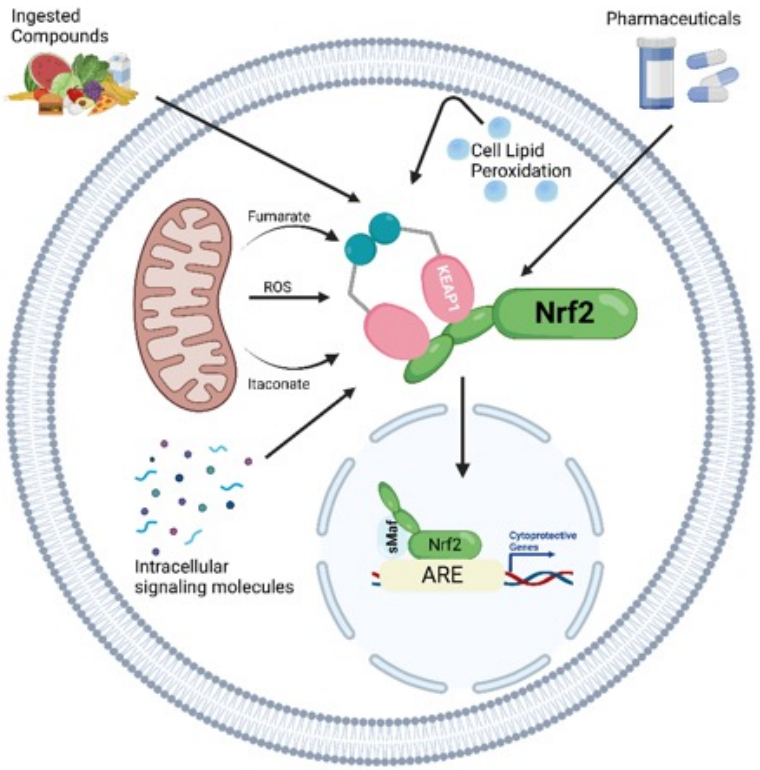
Figure 6:

The KEAP1-NRF2 pathway integrates the sensing of a wide range of cellular stresses to the upregulation of cytoprotective gene expression. Endogenous and exogeneous stress molecules are able to directly bind to reactive cysteine residues within KEAP1, resulting in the stabilization of NRF2 and the upregulation of its cytoprotective transcription program (Adapted and modified from reference 21).

naturally occurring compounds can stimulate various upstream kinases, interfere the Keap1-Nrf2 interaction and/or disturb cellular redox balance, resulting in the activation of the Nrf2 pathway (14).

Compounds like curcumin, present in turmeric, and sulforaphane, found in cruciferous vegetables such as broccoli directly activate Nrf2-Keap1 pathway (22).



Nrf2 and detox proteins

Glutathione, GCLC/GCLM

Glutathione was discovered by J. de Rey-Paihade in 1888 from extracts of yeast and many animal tissues and in fresh egg white. de Rey-Paihade named this substance philothion meaning love and sulfur in Greek. In 1921, Hopkins suggested that the philothion isolated from liver, skeletal muscle, and yeast is a dipeptide consisting of cysteine and glutamate, but these authors overlooked the presence of glycine in philothion possibly due to misinterpretation of the Van Slyke amino N data. Honoring the history of the discovery of philothion, Hopkins named the substance "glutathione" (23). Glutathione, also referred to as GSH, is an endogenous component of cellular metabolism, a tripeptide composed of glycine, cysteine, and glutamic acid. Glutathione conjugation helps contribute to detoxification by binding electrophiles that could otherwise bind to proteins or nucleic acids, resulting in cellular damage and genetic mutations (24). It is found in many tissues at relatively high concentrations,

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namely 1–10 mM in cells, similarly to glucose, potassium, and cholesterol, with a critical role in several physiological processes, such as redox balance preservation, reduction of oxidative stress through detoxification from xenobiotic and endogenous compounds, and immune system modulation. The action of glutathione on oxidative stress has led to speculation on the possible therapeutic role of this molecule for several chronic diseases with altered redox balance (25). GSH is implicated in several functions, including antioxidant defense with reduction of oxidative stress and maintenance of redox balance, metabolic detoxification from xenobiotics and exogenous compounds, cell cycle regulation, and immune system modulation, as well as fibrogenesis (26). Its main role is to shield cellular macromolecules from endogenous and exogenous reactive oxygen species (ROS) and nitrogen ones. In particular, GSH catalytically detoxifies from hydroperoxides, peroxynitrite, and lipid peroxides and directly scavenges various oxidant molecules, like superoxide anion, hydroxyl radical, nitric oxide, and carbon radicals (26; 27). Basically, GSH neutralizes ROS by donating H+ and keeps protein cysteine in active reduced form by offering e–. The ability of GSH as reducing agent is dependent on the GSH/GSSG ratio and total glutathione concentration, which are negatively affected by stress conditions (28). GSH maintains cellular redox status and affects redox signaling, cell proliferation, death and GSH homeostasis is regulated by de novo synthesis as well as GSH redox state. In this way, Nrf2 is critical for maintaining the GSH redox state via transcriptional regulation of GSR and protecting cells against oxidative stress. (29).

Glutamate cysteine ligase (GCL) is an enzyme that plays a critical role in the synthesis of GSH. The catalytic subunit of GCL is a key component of this enzyme. Moreover, the first step in GSH synthesis is carried out by GCL, which is composed of catalytic (GCLC) and modifier (GCLM) subunits (30). Thus, Glutamate-cysteine ligase, also known as gamma-glutamylcystine synthetase is the first rate-limiting enzyme of glutathione synthesis (31).

Heme Oxygenase 1

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that responds to oxidative and/or inflammatory stimuli and it is has the capability to reduce oxidative stress. A major function of HO-1 is to metabolize heme that accumulates in the tissues as a result of red blood cell turnover (32). HO-1 is a Nrf2-regulated gene as it is among those Nrf2-target genes, which is significantly expressed in different cell types (33). Under homeostatic conditions, HO-1 expression is low or absent in most cells and tissues. However, HO-1 is highly upregulated by most cells in response to a vast number of pro-oxidant stimuli and provides protection against oxidative damage.

Expression of HO-1 is largely under the control of the redox-sensitive Nrf2, which binds to the antioxidant response element (ARE) in the promoter region of many antioxidant genes, including HMOX1 (HO-1 encoded by the gene HMOX1 in humans) (34).

Redox signaling

Signal transduction by electron transfer, or redox signaling requires an electrophile (a molecule that attracts electrons) reacting with a nucleophile (a molecule that will give up electrons). Redox signaling involves reactions where an electrophile takes electrons away leaving the nucleophile in a more oxidized state (oxidation reaction). In this situation the electrophile is called an oxidant (35). In summary, oxidation and reduction by loss or gain of an electron induce changes in structural and functional characteristics of molecules, thus modifying signaling processes (36).

Redox signaling is part of the normal physiology of all cells, including flow stimulated growth of endothelial cells, and plays a significant role in pathophysiological responses such as occur in ischemia/reperfusion injury, as well. In contrast with signaling by second messengers like cAMP, signaling by reactive species including involves chemical modification of a target signaling protein cysteine residue rather than non-covalent binding to a protein (35). Events related to redox signaling are triggered under physiological levels of oxidative and nitrosative stresses, and the temporal fluxes in homeostasis are restored through deglutathionylation of redox sensors and increased GSH/GSSG levels (37). The major molecules that participate in redox signaling are the reactive oxygen species (ROS) (36). ROS are compounds that are generated as byproducts of oxidative phosphorylation (aerobic metabolism) in the mitochondria or via cell signaling-induced NADPH oxidases in the cytosol (38). It includes molecules like the superoxide anion, hydrogen peroxide, and hydroxyl radicals, all of which have inherent chemical properties that confer reactivity to different biological targets. ROS is often associated with oxidative stress which suggests they induce damaging in lipids, proteins, and DNA. However, in the past two decades it has become apparent that ROS also serve as signaling molecules to regulate biological and physiological processes. It seems that, early in evolution, nature selected for ROS as a signal transduction mechanism to allow for adaptation to changes in environmental nutrients

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and the oxidative environment. Indeed, in prokaryotes, there are well-described mechanisms whereby ROS directly activate transcription factors for adaption to stress (39).

Redox signaling molecules studies

An 8-week double-blind, placebo controlled human trial demonstrated that consumption of redox signaling molecules (RSM) played a role in the modulation of gene expression tying it to key functions and important pathways in the cells. A few of these genes were showed to be upregulated and connected to the transcription of crucial genes. After a transcriptional profile analysis, it was observed that five genes presented significant fold change in their expression, compared to subjects that did not receive ARS: KCTD12, EGR1, PYROXD1, IRAK3, and CCR10.

Interesting to note that the expression of these genes went back to normal when RSM consumption was stopped, suggesting that continued use of these molecules is required to sustain continued transcriptional profile modulation.

The KCTD12, EGR1, PYROXD1, IRAK3, and CCR10 genes were analyzed using PANTHER (Protein Analysis Through Evolutionary Relationships) and Wikipathways to identify their relationship to different pathways. Some of the pathways are Brain-Derived Neurotrophic Factor Signaling Pathway, Human Thyroid Stimulating Hormone Signaling Pathway, NRF2 Pathway, VEGFA-VEGFR Signaling Pathway2, and others. These and the other pathways related to these genes are crucial for cellular health and function.

Regarding Nrf2 pathway, it was found that this pathway is responsible for turning on and off genes that are essential to produce protective proteins, like Glutathione, that will combat harmful compounds in the body. This pathway is capable of turning gene expression on and off (40; 41; 42).

It is also understood that Nrf2 is a crucial regulator of anti-oxidative response (43; 42), promoting cellular resilience to stress (44; 45). When this pathway is activated, cells are able to be challenged by insults, respond, and restore their functions because activation of Nrf2 pathway promotes cellular homeostasis (46; 47; 48).

Moreover, genes that respond to Nrf2 pathway are involved in the protection of the cells against oxidative stress (43). Actually, Nrf2 pathway is one of the major pathways involved in the adaptation to stress (49; 50). When Nrf2 is activated, more antioxidant proteins are produced in the body to fight oxidative stress (51).

Redox signaling molecules function in AREC32

(human mammary MCF7-derived reporter cell line) AND HEPG2 CELLS (human hepatoma, nontumorigenic cell line)

1- Method

Cell culture on AREc32 and HepG2 cells

The AREc32 cell line is a stably transfected MCF7 cell line that contains a luciferase gene construct under the control of eight copies of the rat Gsta2 ARE. Human liver HepG2 cells possess many of the specialized functions that characterize normal human hepatocytes. Both cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Australia) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Australia), 1% penicillin (Sigma-Aldrich, Australia) in a humidified tissue-culturing environment at 37 °C with 5% CO2. Cultured cells with over 90% confluency were digested with 0.25% trypsin (Thermo Fisher Scientific, Australia) for the following bioassays.

Preparation of electrolyzed water solution

RSM were generated and stabilized according to a patented process of electrolysis using pure salt and water. The presence of RSM was ascertain using appropriate analytical methods. Different RSM samples were prepared and screened for Nrf2 activity (Table 1).

Table 1. RSM samples provided.

Label	Information
Control	0.27% NaCl
V10 v1	Regular, current ARS, sample #1
V10 v2	Regular, current ARS, sample #2
V10 v3	Regular, current ARS, sample #3

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Nrf2 luciferase assay on AREc32 cells

RSM samples were subjected to Nrf2 luciferase assay using AREc32 cells for the examination of Nrf2 activity, where the expression of luciferase is induced via a stably transfected Nrf2 reporter gene construct. Tert-butylhydroquinone (tBHQ), the synthetic food additive potently activates Nrf2, was used as positive control. The control used consisted of the mix of 0.27% NaCl, collected prior to electrolysis.

AREc32 cells were seeded in 96-well plates at a density of 1 x 106 cells/well and were treated with RSM samples when they reached 90% confluence. After 24 h, all the supernatants were taken to fresh 96-well plates for glutathione assay. The cells in the original plates were mixed with triton lysis buffer (20 µL). The cell lysates were obtained by shaking the plate for 10 mins at room temperature and frozen for 20 minutes at -20°C. Finally, the cell lysates were collected (15 µL) and transferred to a 96 well micro-tire white plate. Luciferin buffer (100 µL) was added to the 96-WP and the bioluminescence was recorded within 5 mins [19]. The activation of Nrf2 was calculated by the fold change compared to that of the negative control (cells with medium only).

Cell Viability

Cell viability of AREc32 cells after the treatment of RSM samples for 24h was monitored by the Alamar Blue assay which is widely used for cell viability testing. After the removal of the supernatants, the AREc32 cells were exposed to 100 µL of Alamar Blue dye solution. The plate was subsequently incubated for an additional 2h in a humidified incubator at 37°C. The optical density of each well was determined from measuring excitation at 545 nm and emission at 595 nm using a microplate reader. The absorbance in the control group (cells with medium only) was considered as 100% cell viability, and the cell viability after treatment was calculated by normalizing the absorbance relative to the control to determine the percentage.

Glutathione measurement by high-performance liquid chromatography and fluorescence detection

4-fluoro-7aminosulfonylbenzofurazan (ABD-F) derivatization of glutathione in samples and standards was performed as described in Steele et al., 2012 and Afzal et al., 2023.

A standard solution consisting of 800 mM of cysteine, and 50 mM of cysteine-glycine, homocysteine, and glutathione (GSH) was prepared and then sequentially diluted 2-fold six times in water or DMEM. Each solution was mixed with an equal volume of 1% 5-sulfosalicylic acid containing 1 mM of ethylenediaminetetraacetic acid (EDTA), prior to centrifugation at 14,000 g for 10 minutes at 4oC. The supernatant was subsequently moved to fresh tubes and stored at –80oC, prior to usage in ABD-F derivatization reactions.

Supernatant collected from AREc32 cells after various treatment were removed from freezer storage and allowed to reach room temperature. Vials were placed in a heating block set to 35oC, and 50 µL of the supernatant was then added. Reduction of sulfide bonds was accomplished with the addition of 30 µL of 1 mM tris (2-carboxyethyl) phosphine hydrochloride, and 30 µL of 50 mM N-acetyl cysteine was added subsequently to act as an internal standard. This was followed by 35oC incubation for 5 minutes. 100 µL of borate buffer followed by 30 µL of ABD-F was added to the sample. The sample was then incubated at 35oC for an additional 10 minutes, before the derivatization reaction was ceased through addition of 50 µL of 2 M hydrochloric acid. To remove any particulate matter, the microcentrifuge vials were centrifuged at 14,000 g for 5 minutes at 4oC.

The mixed solutions were then placed into high performance liquid chromatography (HPLC) vials and loaded into the autosampler of a Dionex HPLC system consisting of a WPS-3000 automated sample injector, an ultimate 3000 pumps, ACC-3000 autosampler, column compartment and an FLD-3100 fluorescence detector. The system was equipped with a Luna RP-18 end capped protected by a SecurityGuard C18 Cartridge in a SecurityGuard Cartridge holder supplied by Phenomenex. The Chromeleon 7.0 Chromatography Data System from Dionex was used to control instruments, acquire data, and quantify peak areas.

Western Blot analysis

HepG2 cells were grown in T75 cell flasks until confluence. Cells were then treated with medium, 50 µM tBHQ, V10 v1, V10 v2 at effective concentration for Nrf2 activation. After the incubation for 24 h, cell pellets were harvested by centrifugation at 500 g for 5 min at 4°C. The cell pellets were mixed with radio-immunoprecipitation assay buffer with 1% proteinase inhibitors, and their concentrations were elucidated using PierceTM BCA Protein Assay Kit. The total proteins from each sample at 10 mg/mL were separated by the SDS-PAGE electrophoresis, and then the proteins were transferred to the PVDF membrane by the iBlot 2 gel transfer device. The membranes were incubated with 3% bovine serum albumin (BSA) dissolved in PBST [PBS buffer plus 1% tween 20 for 1h at room temperature. The membranes were incubated with primary antibodies against GCLC, HO-1 overnight at 4°C. GAPDH was used as a loading

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control. The primary antibodies were probed with anti-rabbit HRP conjugated secondary antibodies at room temperature for 2 h. All these anti-bodies were purchased from Cell Signaling Technology. The immunoreactivity bands on the membranes were incubated by the SuperSignal West Pico Plus ECL kit and visualized by the iBright CL750. Specific bands were analyzed, and the intensity was quantified using ImageJ software.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.0 software (GraphPad Software Inc., USA). The data were shown as mean ± standard error of the mean (SEM) from at least three individual experiments. The statistical comparison between groups was conducted by one-way ANOVA with the Tukey test, and $p<0.05$ was considered statistically significant.

2- Results and conclusions

Cell Viability

Cell viability was initially evaluated for a dose-response to understand how AREc32 cells would respond to varying concentrations of signaling molecules. This determined the concentration in which the RSM might be toxic to the cells and identified the optimal dosage range tests as well.

The test assesses the ability of cells to survive and function when exposed to redox signaling molecules (V10 v1 and V10 v2 samples). Results demonstrated that were alive and able to maintain their structural and functional integrity throughout the experiment.

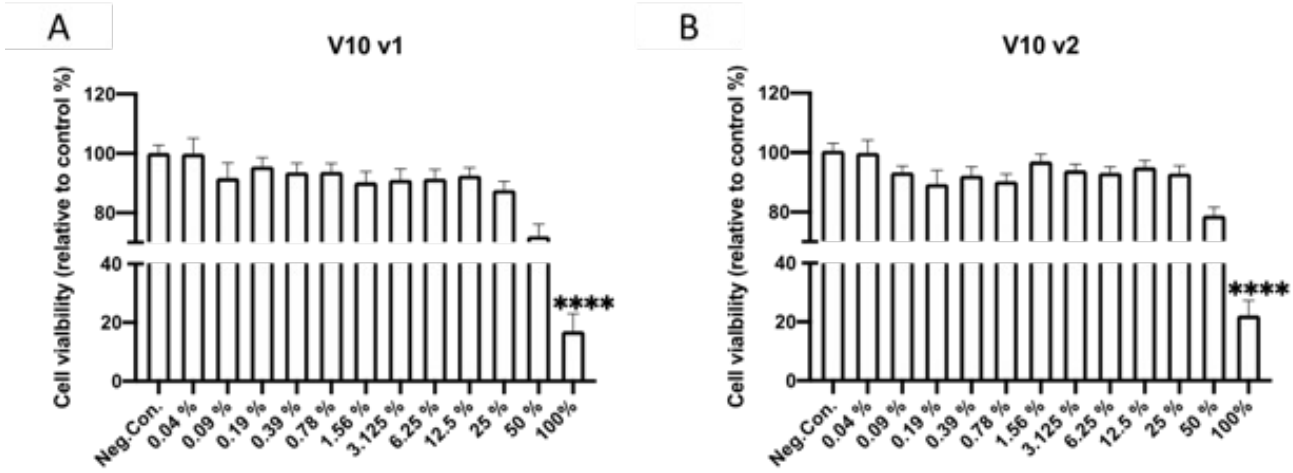

Figure 7:

Cell viability on AREc32 cells (A, B). **** $p<0.0001$ vs. Blank (cells with medium only), by one-way ANOVA analysis in GraphPad Prism 9.

Nrf2 Activation

Most samples showed mild Nrf2 activation (around 2 -fold increase) as opposed to inactivated cells. It was observed a mild yet discernible activation of the Nrf2 pathway in cells treated with ARS. The cells exposed to RSM exhibited an increase in Nrf2 activation which suggests the supplement presented a potential to induce a cellular antioxidant response through the Nrf2 pathway, contributing to cellular defense against oxidative stress and promoting overall cellular health.

Tests on V10 v2 were conducted to compare it with the previous V10 v1 sample to assess the possibility of achieving consistent results, specifically moderate Nrf2 activation. As shown in Figure ABC, the Nrf2 activation looked comparable between V10 v1 and V10 v2 with the maximum Nrf2 activation of 1.63 ± 0.52-fold and 1.69 ± 0.80-fold, respectively. Neither of the solution cause any obvious cell toxicity. Thus, consistent results were obtained.

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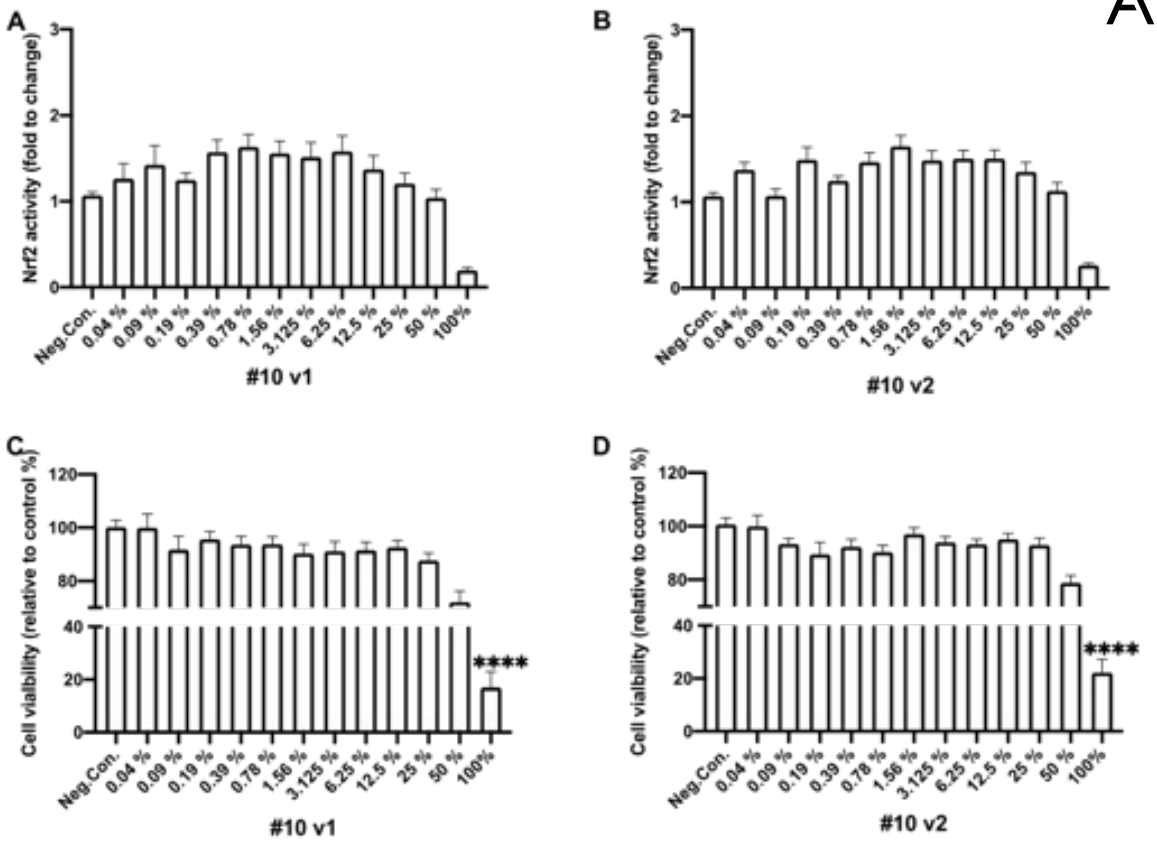
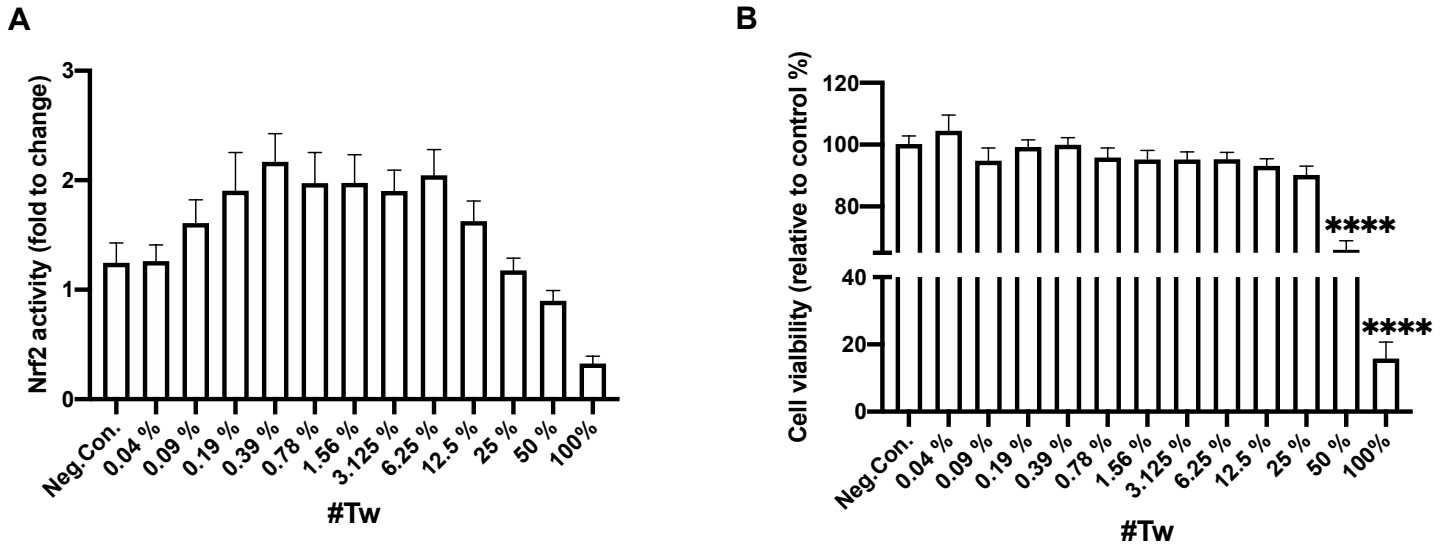


Figure 8:

The Nrf2 activation by RSM V10v1 (A) and V10 v2 (B). The cell viability is shown in (C) and (D). **** p<0.0001 vs. Blank (cells and medium only), by one-way ANOVA analysis in GraphPad Prism 9.

Figure 9:

The Nrf2 activation of V10 v3 (A) and the cell viability (B). **** p<0.0001 vs. Blank (cells and medium only), by one-way ANOVA analysis in GraphPad Prism 9.



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In addition, the Nrf2 activity of the samples has been tested by another research in a different lab to verify the findings. In general, the research outcomes are similar. The detailed results and the comparison between the two researchers for the Nrf2 activation is shown in Table 2.

Label	NICM's Nrf2 results	SOM's Nrf2 results
Blank	1	1
V10 v1	1.63±0.52	2.25±0.19
V10 v2	1.69±0.80	2.23±0.23
V10 v3	2.04±0.91	3.5
Positive control	12.43 ± 2.93	16.89 ± 3.62

Table 2. RSM of maximum Nrf2 effects at National Institute of Complementary Medicine (NICM) and School of Medicine (SOM). Results showed represent the fold change in Nrf2 activation compared to control (Blank) where no treatment was added to the culture. V10 v1, V10 v2, and V10 v3 demonstrated a mild yet discernible activation of Nrf2 pathway in the cells treated with RSM.

In **Table 2**, two separate laboratories have, independently, achieved comparable results when analyzing the same set of samples. Particularly, the Nrf2 activity consistently appeared in #10. These consistent findings have led to the selection of these samples for further mechanistic experiments aimed at investigating Nrf2-regulated antioxidant potential.

The Nrf2 activation looked comparable between V10 v1 and V10 v2 with the maximum Nrf2 activation of 1.63 ± 0.52-fold and 1.69 ± 0.80-fold, respectively. Neither of the solution cause any obvious cell toxicity. V10 v3 caused a maximum Nrf2 activation of 2.04 ± 0.91-fold to control at concentration of 6.25% in media, without causing any obvious cell toxicity.

Increased Nrf2-regulated GSH expression

To investigate a possible increase in GSH production by redox signaling molecules, the extracellular levels of GSH and the derived thiol CysGly were determined in AREc32 cells by HPLC with fluorescence detection. Activation with V10v2 resulted in a 1.52-fold increase. We also noticed a significant decrease in cysteine and CysGly productions by V10 v2. These results suggested that V10 v2 induced the metabolism to increase expression of GSH.

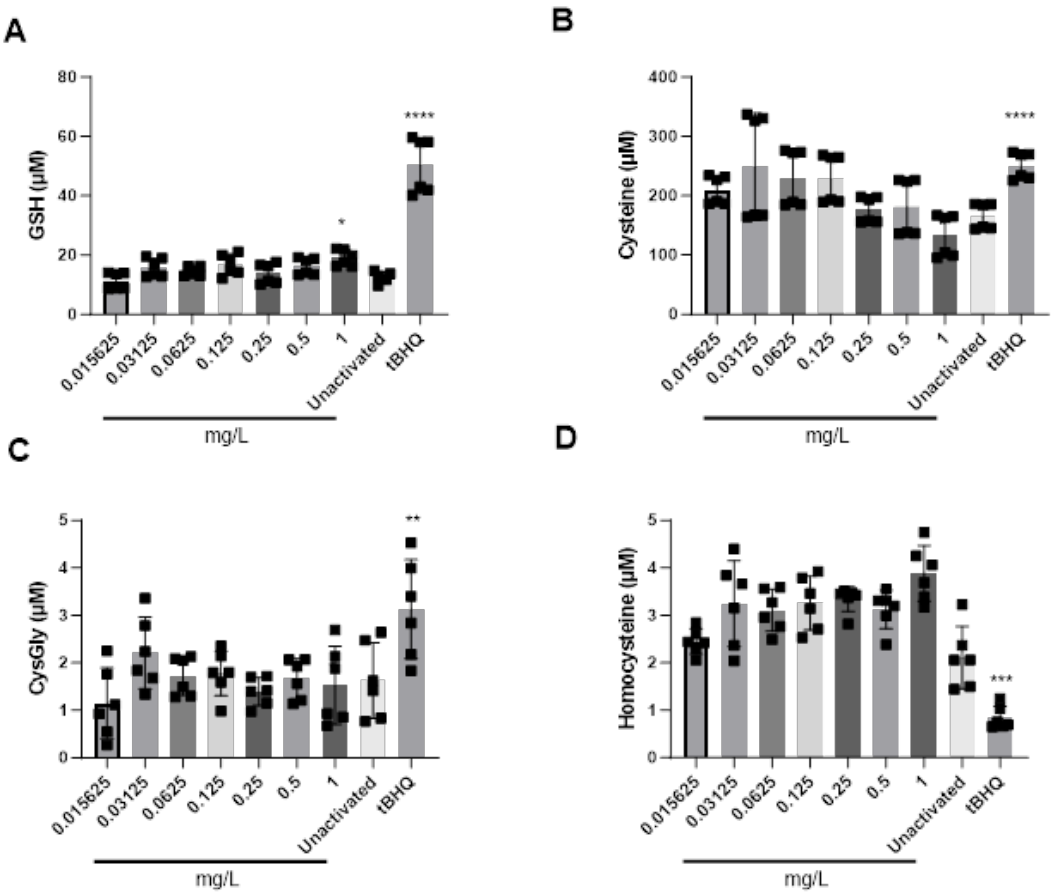

Figure 10:

Expressions of (A) GSH, (B) cysteine, (C) cysGly and (D) homocysteine induced by #10v2 on AREc32 cells. Unactivated represents cells with medium only. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with un-activated cells by one-way ANOVA.

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Increased Nrf2-regulated GCLC and HO-1 expressions

To complement the Nrf2 luciferase activity measurement, the effect of V10 v2 on the expression of the Nrf2 regulated target proteins GCLC and HO-1 was investigated by Western blot. A human liver cell line, HepG2 cells, was used for these experiments. HepG2 is a human hepatoma cell line that is commonly used in drug metabolism and hepatotoxicity studies.

The positive control tBHQ (50 μM) significantly upregulated the expression of GCLC and HO-1 (Figure 11 and 12). Western blot analysis for the antioxidant proteins expression GCLC demonstrated that #10 v2 exhibited higher expressions of this protein compared to blank (negative control, $p<0.01$). In addition, V10 v2 (0.25 mg/L) induced significantly higher HO-1, suggesting prominent antioxidant activities.

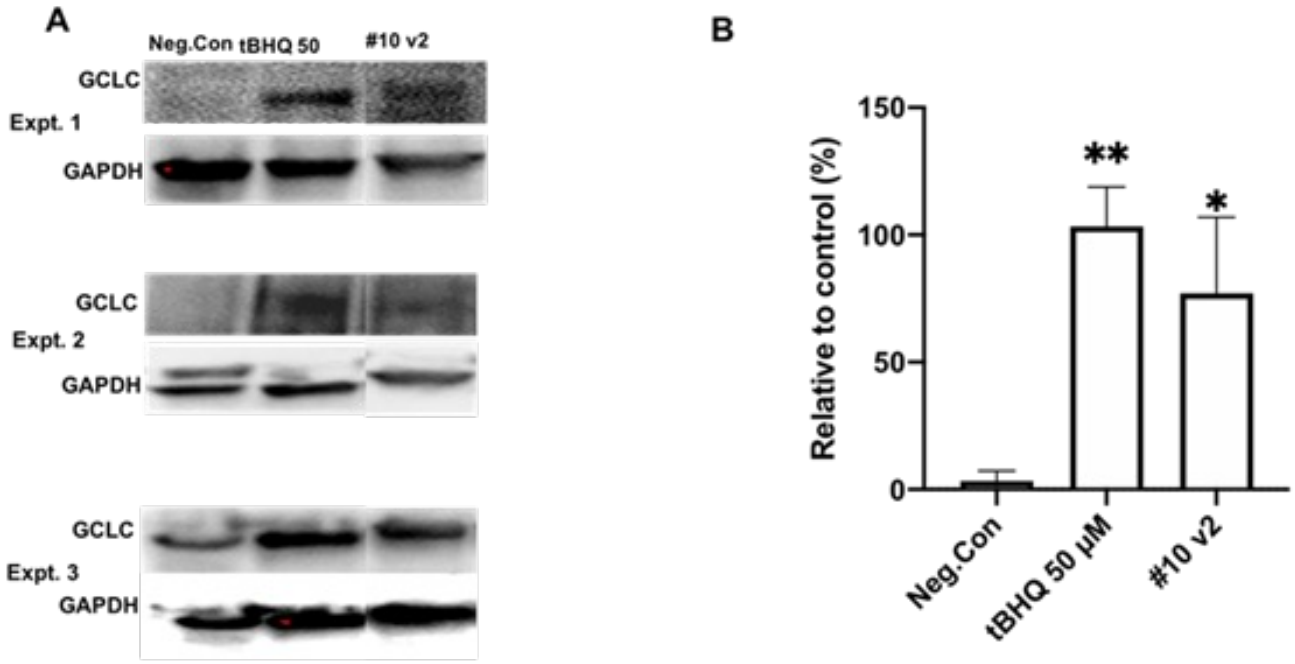

Figure 11:

GCLC protein expression by Western blot analysis. HEGP2 cells were cultured in T75 cell flasks and were on the treatment for 24 hrs. (A) Protein expression levels of GCLC with n=3 experiment. (B) The data was analysed by ImageJ and input to Graphpad Prism 9. *$p<0.05$, ** $p<0.01$ vs. negative control (cells with medium only).

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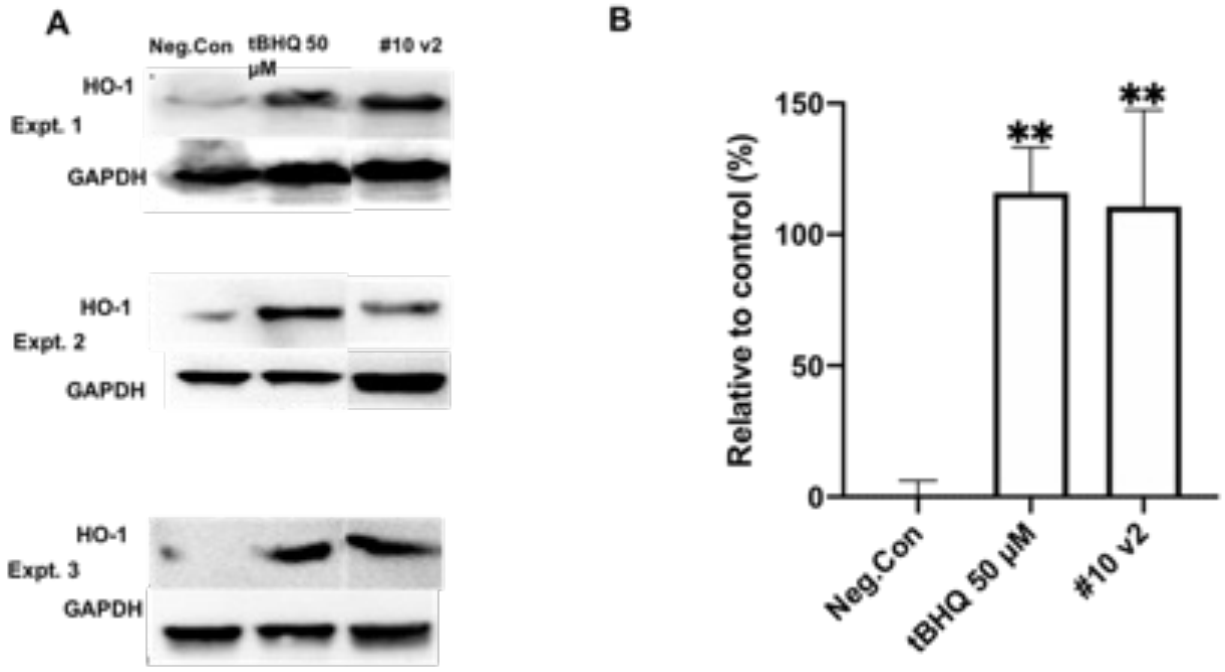

Figure 12:

HO-1 protein expression by Western blot analysis. HEGP2 cells were cultured in T75 cell flasks and were on the treatment for 24 hrs. (A) Protein expression levels of HO-1 with n=3 experiment. (B) The data was analysed by ImageJ and input to Graphpad Prism 9. *p<0.05, ** p<0.01 vs. negative control (cells with medium only).

In summary, cells that were exposed to ARS showed an upregulation of GCLC and HO-1 compared to control, meaning they are increasing the rate at which these proteins are being produced.

Increased GCLC expression is a part of the cell's adaptive response to counteract oxidative stress and maintain redox balance. GCLC catalyzes the formation of a dipeptide, gamma-glutamylcysteine, which is a precursor for the subsequent synthesis of GSH. As GSH plays a vital role in neutralizing ROS, detoxifying harmful substances, and protecting cells from oxidative stress-induced damage, upregulation of GCLC might serve as a protective mechanism to enhance the cellular antioxidant capacity and promote cell survival under conditions of increased oxidative stress.

The upregulation of HO-1 can be considered a protective and adaptive response to various cellular stressors, including oxidative stress, and a part of the cellular defense mechanisms against stress that can contribute to maintaining cellular homeostasis and promoting cell survival under challenging conditions.

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Redox signaling molecules function in FEK4 cells

(human primary foreskin fibroblasts)

1- Method

Cell Culture and MTT Assay

FEK4 cells were cultured 48 hours and 15% serum media overnight. Washed with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% triton for 15 min, blocked with 2% BSA for 45 min and cultured with Nrf2 polyclonal antibody (1:100) in 0.1% BSA at 4 °C overnight, then stained with Alexa fluor plus 488 (1:2000) in 0.1% BSA for 45 min and a drop of DAPI prior imaging.

Human primary skin fibroblasts FEK4 cells were seeded on day 0. After 48 h in culture, the medium was changed and redox signaling molecules were added to the culture at the dilution ratio of 1:1 in media. After 24 hours in culture in the presence of RSM, the cells were fixed and proceeded for immunocytochemistry with Nrf2 antibody and nuclear marker DAPI.

Cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used method to assess cell viability and proliferation in vitro. Briefly, cells are cultivated in medium (in the presence or not of RSM) under standard conditions (37°C, 5% CO2) until they reach the desired confluence. The culture medium is removed, and the cells washed with PBS. MTT solution is added to each and incubate for 2-4 hours at 37°C. The MTT is reduced to formazan by metabolically active cells. The MTT was carefully aspirated, replaced with DMSO to dissolve the formazan crystals, and pipetted up and down to mix. After mixing, the absorbance of each well was measured at a wavelength around 570 nm with a microplate reader. The absorbance correlates with the number of viable cells. Data collected was analyzed to determine and calculate the viability percentages.

Glutathione Assay

Cells are cultivated in regular medium until confluency was achieved. The media was removed and replaced by a fresh media containing RSM at 1:1 ration containing 50 µM of buthionine sulfoxide (BSO). Controls were cultivated without BSO, which is a well-known inhibitor of the gamma-glutamylcysteine synthase, which is the first step of GSH synthesis. After 24 hours in culture, the glutathione assay was performed using Bio-Rad (Bradford assay). Bradford protein assay is a simple and accurate colorimetric procedure for determining the concentration of protein (glutathione in this case) in solution. When the dye binds to protein, it is converted to a stable unprotonated blue form (Amax= 595 nm). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader.

Nrf2 activation /nuclear translocation

Human primary skin fibroblasts FEK4 cells are seeded and cultivated in medium under standard conditions (37°C, 5% CO2). After cells reached the desired confluence RSM are added to the culture at a dilution rate of 1:1 in media. Cells are cultivated in the presence of RSM for 24 h. After that period, the medium is removed, the cells washed with phosphate buffer saline (PBS) and fixed in formaldehyde. Fixed cells are permeabilized with Triton X-100 and non-specific binding sites were blocked BSA. Cells are incubated with primary antibody (anti-Nrf2), then washed with PBS, and incubated again with secondary antibody. Cells are also stained with DAPI, for nuclear localization. Cultured cells are mounted on microscope slides using mounting medium and stained cells are visualized using a fluorescence microscope.

2- Results and conclusions

Cell Viability

Data obtained with MTT assays showed that overnight treatments of cells with redox signaling molecules at dilution ratio used, did not decrease cell viability of treated cells, when compared to untreated control (Figure 13).

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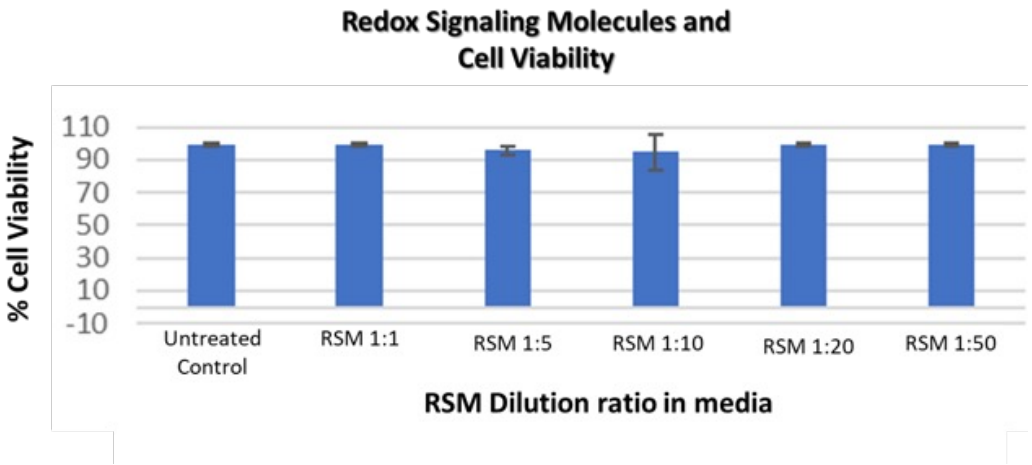

Figure 13: Data expressed as percentage cell viability of cells treated with different concentrations of redox signaling molecules compared to untreated control (Ctrl) set as 100%. Data presented as an average of at least 3 independent experiments (n=3) +/- standard deviations sown as error bars.

Glutathione Assay

Cells that were exposed to redox signaling molecules overnight showed increased the intracellular level of GSH by 40% (2.3-fold increase, when compared to untreated control) compared to control.

Treating cells with BSO alone reduced the intracellular GSH level to 0.5-fold of untreated control fibroblasts. However, in BSO-treated cells, redox signaling molecules showed capacity to restore the intracellular GSH level to up to 0.8-fold of the untreated control fibroblasts. BSO is a known inhibitor of the gamma-glutamylcysteine synthase, which is the first step of GSH synthesis. Addition of redox signaling molecules showed to restore GSH production in the cultured cells (Figure 14).

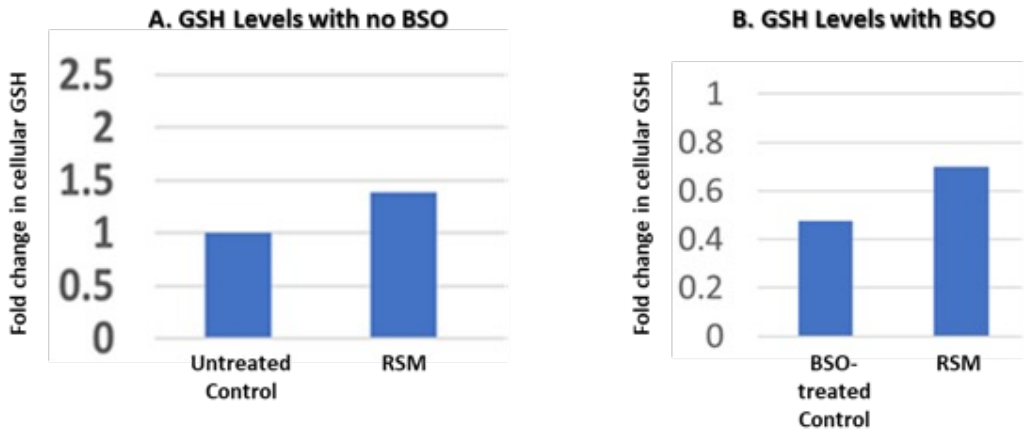

Figure 14: A. Glutathione (GSH) levels on cells exposed to redox signaling molecules (Formulation 1) in comparison to untreated cells (control). B. Cell treated with Buthionine Sulphoximine (BSO) and redox signaling molecules showed increased expression of GSH compared with BSO-treated cells. Data were calibrated as nanomoles of GSH per mg of protein and then plotted as fold change in GSH compared to untreated control (Ctrl), which was arbitrary set as 1. Data presented as an average of at least 3 independent experiments (n=3) +/- standard deviations sown as error bars.

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Nrf2 Translocation to the Nucleus

Figure 15 shows that redox signaling molecules were able to promote Nrf2 translocation to the nucleus (column 3, aqua coloration of the nucleus) when cells were exposed overnight to the treatment. These results are consistent with the superior abilities of redox signaling molecules to increase the intracellular GSH levels.

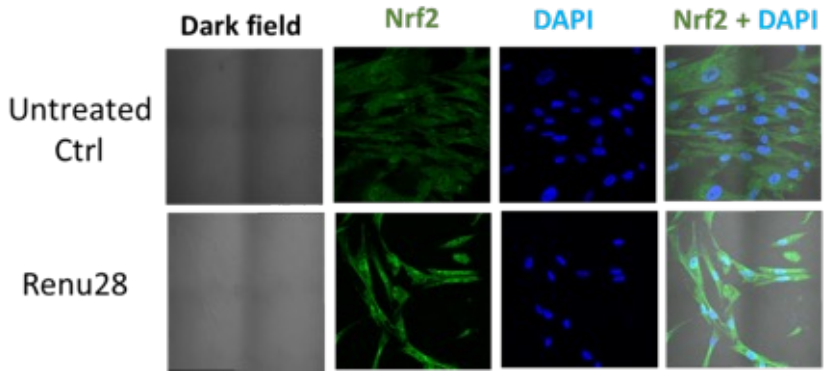

Figure 15: Immunofluorescence analysis of human primary skin fibroblasts FEK4 cells treated with redox signaling molecules (RSM) or untreated (control, Ctrl). Nrf2 translocation to the nucleus. Column 1, immunocytochemistry to localize NRF2 proteins. Column 2, nuclear staining with DAPI. Column 3, merged image of NRF2 protein and DAPI. Data are representative of 3 independent experiments. Mean Fluorescence intensity (MFI). Quantification of Nrf2 translocation to nucleus with image J software.

RSM and Nrf2 – closing remarks

The investigation into the role of redox signaling molecules (RSM) in AREc32 and HepG2 cells, as well as FEK4 cells, has yielded insightful findings regarding their impact on pathways that protect cells from oxidative stress. The experiments provided understanding of Nrf2 cellular responses to RSM and also demonstrated the capability of RSM to upregulate glutathione production on the cells.

The results indicated that RSM containing in V10, induces a mild yet discernible activation of the Nrf2 pathway in AREc32 and HepG2 cells, contributing to cellular defense against oxidative stress. The consistent Nrf2 activation across different sample lots, as verified by independent laboratories, strengthens the reliability of our findings. Additionally, the investigation into Nrf2-regulated antioxidant potential revealed increased expressions of GCLC and HO-1, suggesting a cellular adaptive response to enhance antioxidant capacity (43).

Furthermore, our exploration extended to FEK4 cells, where redox signaling molecules demonstrated no cell toxicity or impairment on cell viability. Results showed that RSM were able to increase and/or restore intracellular glutathione levels, after BSO treatment, as well as promoting Nrf2 translocation to the nucleus. These observations suggest the potential of RSM in supporting cellular health and antioxidant defense mechanisms.

The positive results obtained from different cell lines and assays have provided a comprehensive perspective on the multifaceted impact of redox signaling molecules on cellular functions. These findings contribute to the growing body of knowledge in the field of redox signaling and may pave the way for further studies to unravel the underlying pathways involved in RSM response to cellular stress.

These studies demonstrated consistent and promising results connecting the role of RSM to cellular resilience and antioxidant defense. As a matter of fact, this research may set the stage for future investigations, potentially leading to the development of innovative approaches for cellular health enhancement and oxidative stress management.

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