IN VITRO EFFECTS OF GOLD PARTICLES ADDED TO REDOX SIGNALING MOLECULES

Experiments conducted by Dr. Charareh Pourzand

University of Bath, UK

Highlights

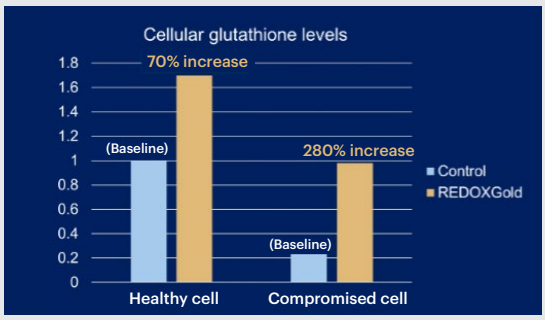
Therapeutic potential: Overall, the findings from this study provide strong evidence for the efficacy of redox signaling molecules gel containing gold (RSM-GG) formulations in promoting antioxidant responses, enhancing recovery, and protecting from oxidative damage. The dual action of increasing GSH levels and activating the Nrf2 pathway highlights the RSM-GG potential effect in cooling areas of discomfort and other applications where oxidative stress is a contributing factor.

Enhanced antioxidant capacity: The GSH/BSO assay demonstrated that RSM-GG formulations significantly increased intracellular GSH levels, enhancing cellular antioxidant capacity. This effect was particularly notable when RSM-GG was able to restore GSH levels by up to 280% (Graphical highlight 1) in BSO treated cells, counteracting the GSH-depleting impact of this chemical.

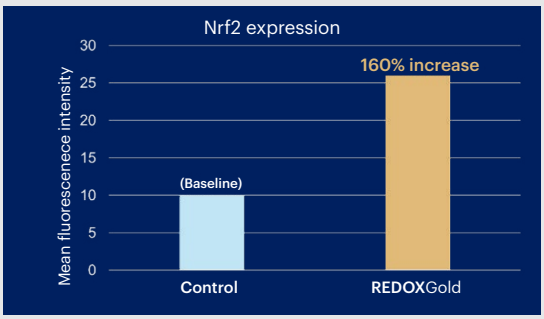
Nrf2 pathway activation: The Nrf2 activation assay showed that RSM-GG formulations promoted Nrf2 translocation to the nucleus, with an increase of up to 160% (Graphical highlight 2) compared to untreated controls. This activation is crucial for upregulating the detoxifying and antioxidant enzymes, bolstering cells' defense mechanisms against oxidative stress and aiding in cellular recovery.

Increased NQO1 expression: The NQO1 synthesis assay revealed that RSM-GG formulations significantly increased the expression of NQO1, with levels reaching up to 150% compared to untreated controls. This supports the activation of the Nrf2 pathway and enhances the cellular antioxidant response.

Safety and non-toxicity: The MTT viability assay confirmed that RSM-GG formulations, at the specified dilution ratios, did not decrease cell viability compared to untreated controls, indicating their non-toxic nature and safety for use under experimental conditions.



Graphical highlight 1



Graphical highlight 2

1. Introduction

1.1. Gold, a transition metal: history and facts

Gold, a member of Group 11 (d-block) in the periodic table (Figure 1), is a dense, lustrous, and highly malleable transition metal (1, 3). Its unique properties, including a resistance to oxidation (tarnishing), have captivated humans for millennia (2, 6). Gold exhibits several unique properties that contribute to its high value and versatility. It is highly resistant to corrosion and oxidation, ensuring it remains untarnished over time. The rarity and distinctive appearance of gold likely fueled its association with value and power. Gold's high density (19.3 g/cm³) and non-magnetic nature further distinguish it from other metals,

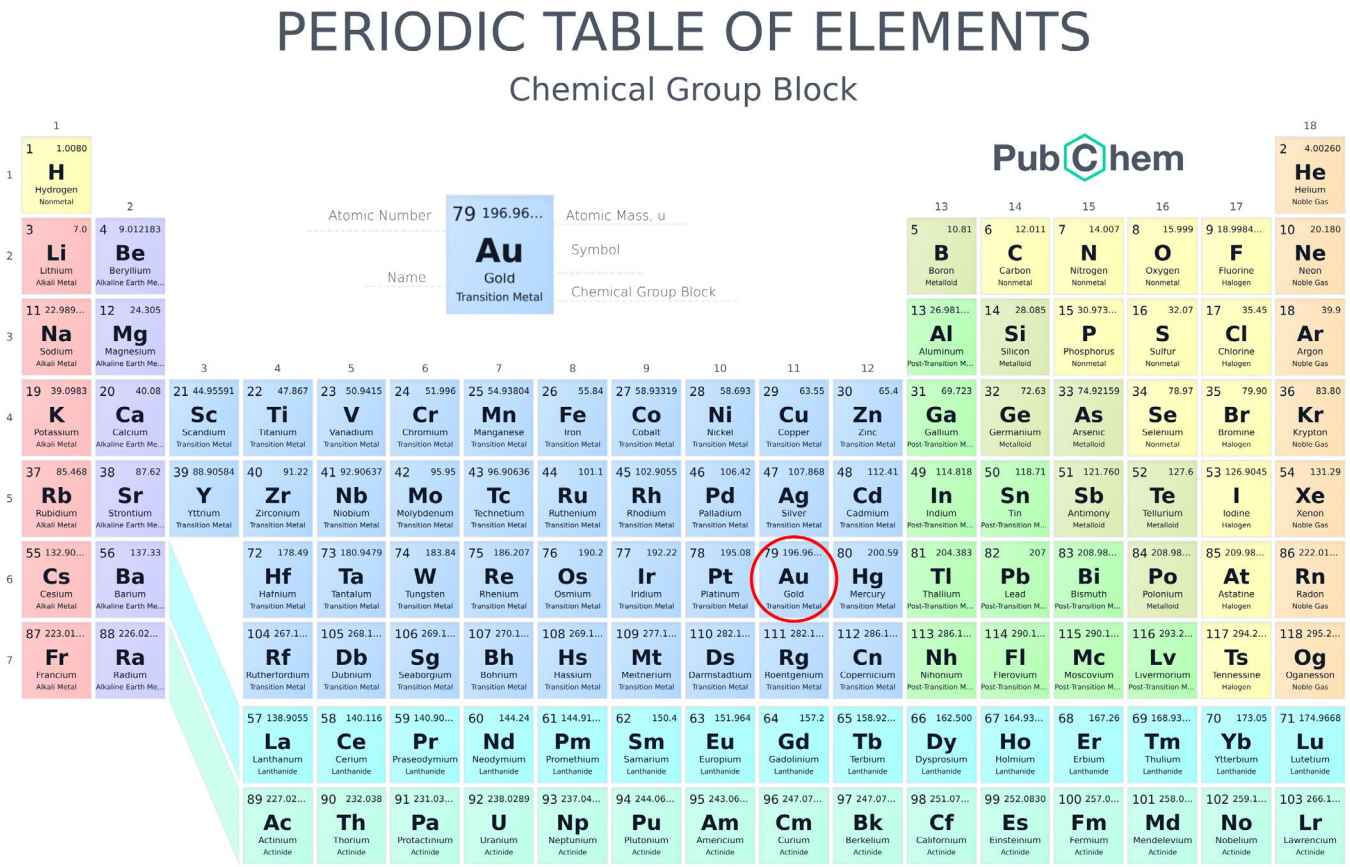


Figure. 1: The Periodic Table of Elements (3)

contributing to its unique physical and chemical properties (3, 5). In biological systems, gold compounds and ionic gold play significant roles. Gold compounds, such as gold salts, have been used in medicine for their anti-inflammatory and anti-rheumatic properties. For instance, gold sodium thiomalate and auranofin are used in the treatment of rheumatoid arthritis due to their ability to modulate immune responses and reduce inflammation (4). Ionic gold (Au^{3+}) can influence various biochemical pathways. These interactions can lead to therapeutic effects, such as inhibiting the activity of certain enzymes involved in inflammatory processes (7). The unique properties of gold, combined with its biocompatibility, make it a valuable material in medical and biological applications (4, 7).

1.2. Historical use of gold in medicine

Gold has held a prominent position in medical practice for millennia. Its perceived therapeutic properties can be traced back to 2500 BC in China, where it was used to treat ailments like smallpox, skin ulcers, and joint diseases. Ancient Chinese physicians believed gold possessed restorative powers and was harmless (1, 6). During the Middle Ages, gold-coated pills and "gold waters" were popular, with alchemists using them to treat sore limbs, foreshadowing the future use of gold in arthritis treatment (1, 6).

South Indian Siddhars, revered for their alchemical knowledge, are credited with developing various gold-based medicines like "thanga parpam" and "thanga chendooram" (8).

1.2.1. The rise of gold therapy

The Renaissance period saw a renewed interest in gold therapy with figures like Paracelsus, a renowned alchemist, advocating for its use in treating various diseases and even achieving immortality (7). This period witnessed the rise of "drinkable gold" in the 16th-century French court and the publication of dedicated texts by physicians like Alexandre de la Tourette and Jean Beguin (9).

1.2.2. Modern developments in gold therapy for rheumatoid arthritis

The 20th century ushered in the modern era of gold therapy. Surgeons began using gold implants near inflamed joints for pain relief, and in 1929, Jacques Forestier, a French internist, discovered the effectiveness of gold salt injections in alleviating rheumatoid arthritis (RA) symptoms (10).

Gold therapy, also known as chrysotherapy or aurotherapy, involves administering gold salts via injections or oral tablets. Gold compounds like auranofin (AUR) exhibit anti-inflammatory properties by suppressing the production of pro-inflammatory molecules associated with RA (11). Although initially used for various diseases, research gradually revealed the effectiveness of gold therapy specifically for RA.

1.3. Mechanisms of action

Gold salts exert their therapeutic effect in RA through several key mechanisms (10):

- **Antioxidant activity:** Gold scavenges reactive oxygen species (ROS) and inhibits enzymes contributing to oxidative stress, a crucial factor in RA.
- **Lysosomal enzyme inhibition:** Gold can inhibit the activity of lysosomal enzymes involved in tissue damage during inflammation.
- **Reduced monocyte adhesion:** Gold therapy limits the adhesion and infiltration of monocytes into the synovium (joint lining) by inhibiting specific molecules like E-selectin and prostaglandin synthesis. This reduces inflammation within the joint.
- **T lymphocyte regulation:** Gold therapy can prevent T lymphocyte proliferation by inhibiting protein kinase C and altering communication between T lymphocytes and monocytes, thereby dampening the immune response.
- **Collagenase inhibition:** Gold suppresses the production and activity of collagenase, an enzyme that breaks down collagen, a major component of cartilage. This helps preserve joint integrity.

These combined actions ultimately lead to reduced joint pain and swelling, along with a decrease in inflammatory markers in the blood. While gold therapy was once a mainstay for RA, the development of alternative medications has led to its decline in use. However, its historical significance and unique mechanisms of action continue to hold scientific interest, potentially paving the way for future therapeutic applications.

1.4. The renaissance of gold

Recently, research into gold-based drugs for a range of human diseases has seen a renaissance. Both old and newly developed Au(I) and Au(III) compounds have been utilized and designed to target cellular components involved in the onset or progression of cancers, rheumatoid arthritis, and viral and parasitic diseases. Additionally, new disease targets for gold compounds have been identified, providing insights into their mechanisms of action and the molecular pathophysiology of human diseases (12).

AUR is currently enrolled in clinical trials for potential repurposing against cancer. The drug mainly targets the anti-oxidative system catalyzed by thioredoxin reductase (TrxR), which protects the cell from oxidative stress and death in the cytoplasm and the mitochondria (13). It inhibits the induction of proinflammatory proteins and their mRNAs by the inflammatory stimulants, cyclooxygenase-2 and inducible nitric oxide synthase, and their upstream regulator, NF-kB. AF also activates the proteins peroxyredoxin-1, Kelch-like ECH-associated protein 1 (Keap-1), and NF-E2-related factor 2 (Nrf2), and inhibited TrxR, all of which are involved in oxidative or electrophilic stress under physiological conditions. Ongoing trials are exploring the repurposing of AF as a controlled-risk treatment for cancers and infectious diseases, including COVID-19. Additionally, new gold compounds are being developed as potential anti-cancer and anti-infection agents (11).

Gold(I) compound has anti-inflammatory properties; it reduces IL-6 expression via inhibition of the NF-kB-IL-6-STAT3 signaling pathway. Also, by inhibiting redox enzymes such as TrxR, AUR increases cellular oxidative stress and promotes apoptosis. Interestingly, in vitro tests showed that AUR treatment resulted in significant reduction in SARS-COV-2-induced cytokines in human cells. It was reported that this gold-containing drug reduced by 95% SARS-CoV-2 RNA in infected human cells in vitro and decreased SARS-CoV-2-induced cytokine expression, including IL-6. Additionally, it is proposed that AUR can mitigate SARS-COV-2 infection and lung damage due to its anti-viral and anti-inflammatory properties (14, 35).

1.5. Gold and dermal penetration

Human skin is the largest organ of the human body, forming an effective physical barrier against environmental conditions while simultaneously functioning as a semipermeable membrane that helps maintain proper moisture within the body. Physically, the skin protects from external threats such as infectious agents, chemicals, systemic toxicity, and allergens. Internally, the skin helps to maintain homeostasis and protects from enhanced loss of water from the body. (Figure 2) (15). This barrier function of the skin is based on the stratum corneum, located in the uppermost skin. Stratum corneum has cells called corneocytes, surrounded by multilamellar lipid membranes, which are composed of cholesterol, free fatty acids and ceramides (Figure 3) (16).

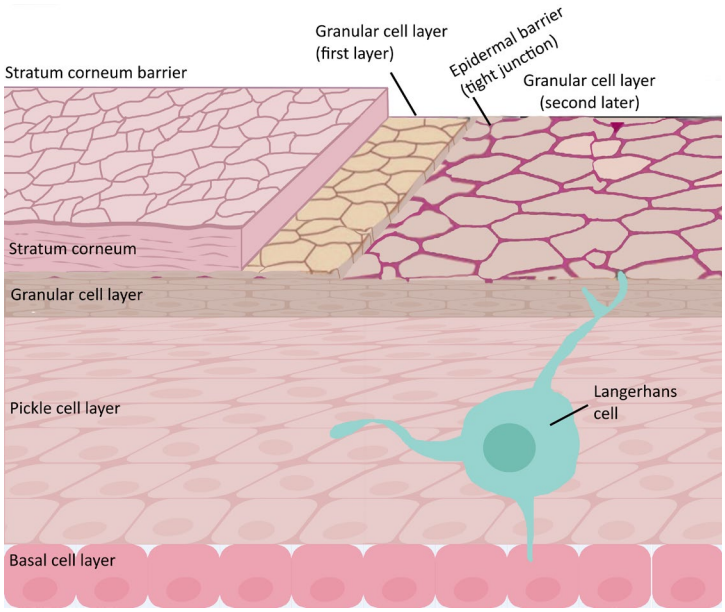


Figure 3: Structure of the stratum corneum [Modified from Katoh, N. et al., 2020 (17)].

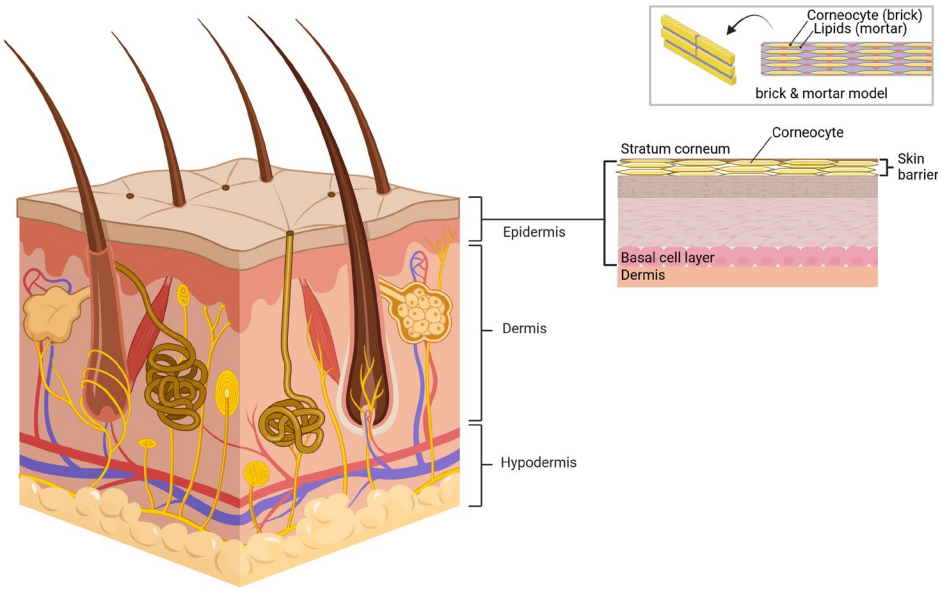


Figure 3: Structure of the stratum corneum [Modified from Kahraman, E. et al., 2019 (18)]

Specifically, literature about dermal penetration of gold ions or large gold particles in skin is very scarce. There is a higher amount of research available nowadays in regard to smaller particles, or gold nanoparticles (AuNPs). They are widely used for cellular imaging and have become a significant tool in the field of medicine due to their unique properties and versatility for drug delivery, cancer therapy and others. By definition, nanoparticles are ultrafine particles with dimensions measured in nanometers (nm), typically ranging from 1 to 100 nm (19). Particles larger than 100 nm are generally not considered nanoparticles and fall into the category of larger particles, often referred as microparticles (20, 21). Crucially, these large particles, including large gold particles, are unable to penetrate the skin barrier. This emphasizes that large particle size effectively prevents dermal penetration.

1.6. Gold and activation of Nrf2

Gold-containing compounds, such as AUR, have notable effects on the Nrf2-sMaf signaling pathway, which plays a crucial role in cellular defense mechanisms against oxidative stress and inflammation (23).

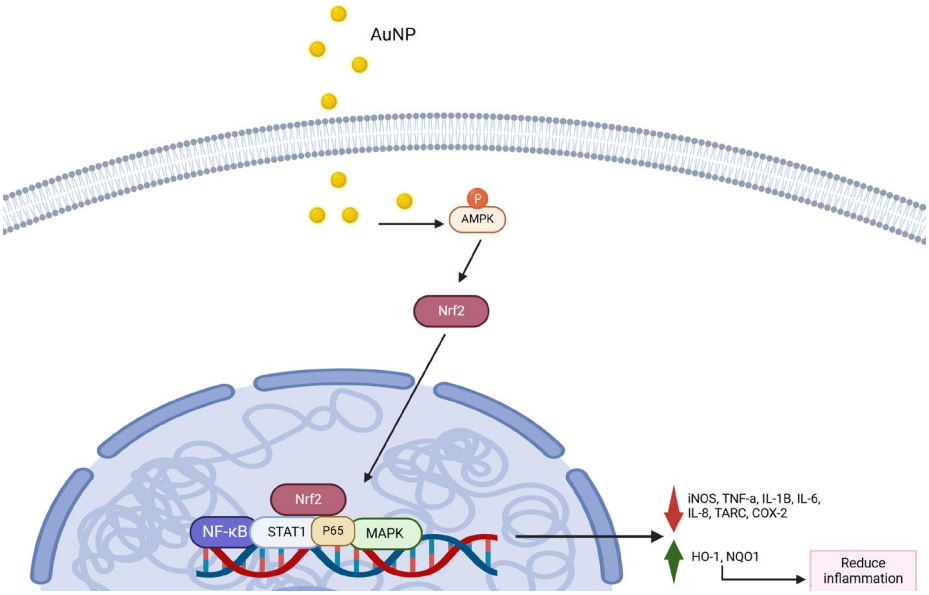
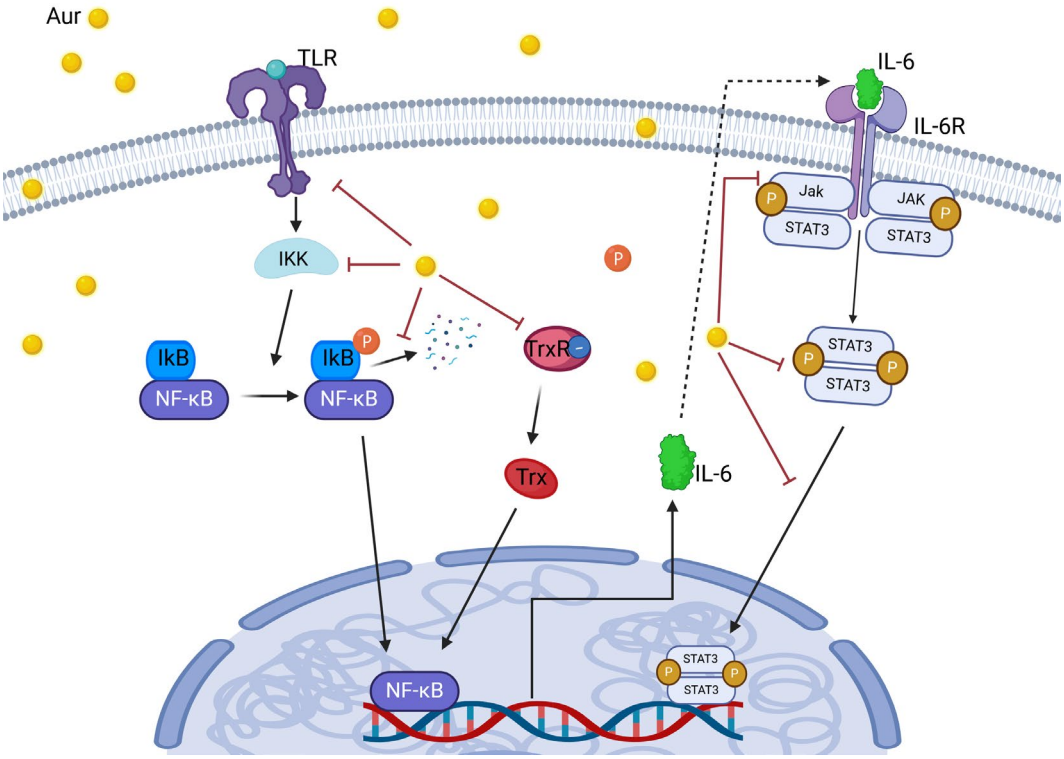


Figure 4. Gold nanoparticles (AuNPs) activates AMPK/Nrf2 signaling, promotes quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1) expression, and reduces inflammation. [Modified from Aili, M. et al., 2023 (27)]

Gold compounds can activate Nrf2 by modifying its inhibitor, Keap1. This modification prevents Keap1 from targeting Nrf2 for degradation, allowing Nrf2 to accumulate and translocate to the nucleus. The ability of AuNPs to directly activate the Nrf2 pathway renders them good candidates for treatment of disorders in which the up regulation of Nrf2 is beneficial, specifically for topical treatments of inflammatory skin diseases (24). AuNPs enhance the levels and nuclear translocation of the Nrf2 protein and Bach1 export/tyrosine phosphorylation, leading to Nrf2 binding to the heme oxygenase 1 (HO-1) enhancer promoter region E2 to drive its expression in endothelial cells (25). Additionally, AUR also leads to HO-1 upregulation by activating Keap1/Nrf2 signaling via Rac1/iNOS induction and MAPK activation (26) (Figure 4).

An investigation into transcriptional regulation by antirheumatic gold(I) drugs, found that they selectively activated the binding of the heterodimeric bZip transcription factor Nrf2 and small Maf to the MARE/ARE, resulting in the induction of oxidative stress-responsive genes, which may be an important pharmacological effect of these drugs for the treatment of RA. (28). Additionally, gold(I) drugs have been reported to down-regulate the expression of various genes that promote inflammation. These include genes for collagenases, the interleukin-2 (IL-2) receptor, cytokines (such as IL-1, IL-2, IL-6, IL-8, and TNF-alpha), and producers of chemical mediators like cyclooxygenase-2 and inducible nitric-oxide synthase. This down-regulation is thought to be mediated by the inhibition of AP-1 (Jun/Fos) and NF-κB transcription factors. In fact, gold(I) drugs have been shown to selectively inhibit the DNA binding activities of AP-1 and NF-κB in vitro, as well as inhibit IκB kinase activity in vivo (29).



Auranofin, a gold-based compound, works by inhibiting key processes, including the dimerization of TLR4, activation of IKK, phosphorylation of JAK1 and STAT3, translocation of STAT3 to the nucleus, and the activation of TrxR, ultimately reducing NF-κB transactivation and inflammation. [Modified from Sonzogni-Desautels et al, 2021 (30)].

Mechanisms have demonstrated that AuNPs and AUR may reduce the expression of pro-inflammatory cytokine by inhibiting MAPK, NF-κB, JAK/STAT, and IKK-α/β signaling pathways and downregulating their downstream pro-inflammatory mediators, such as iNos, TNF-α, IL-1β, IL-6, IL-8, TARC, COX-2. Meanwhile, AuNPs activates AMPK/Nrf2 signaling, promotes quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1) expression, and reduces inflammation (Figures 4 and 5) (27).

2. MATERIAL AND METHODS

2.1. Redox signaling molecules gel containing gold

A proprietary formulation, referred to as electrolyzed water containing redox signaling molecules (EW), was utilized in this study as the base to produce the redox gold gel. EW was first designed and patented under the United States Patent number 8,663,705 B2 (March 4th, 2014): Method and Apparatus for Producing a Stabilized Antimicrobial Non-Toxic Electrolyzed Saline Solution Exhibiting Potential as a Therapeutic. Lately, the method described in the patent was significantly improved and provided a competitive advantage, therefore the patent owner chose to keep the improvements over the original patent confidential, protecting them as a trade secret. To produce the redox signaling molecules gel (RSM-GG) containing gold, a well-calibrated EW was produced through an electrochemical process. Following the electrolysis, sodium magnesium fluorosilicate, gold metal micro-powder (200–700 nm particles), and gold flakes were added. The compounds were well mixed and bottled. Specific details regarding the composition and manufacturing process of the RSM-GG are proprietary and protected under trade secret regulations. For the tests, different RSM-GG formulations were individually dissolved in DMEM before the tests. The solutions were then added in different ratios directly into the culture containing the cell monolayer.

2.2. Cytotoxicity determination of the RSM-GG

To assess cytotoxicity of the different formulations of the RSM-GG in the presence of gold, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were conducted for each formulation. The MTT assay is a colorimetric assay used to assess cell metabolic activity. It measures the ability of cells to reduce the yellow MTT dye to a purple formazan product. This reduction is carried out by NAD(P)H-dependent cellular oxidoreductase enzymes, which are active in viable cells (31). All tests were conducted using human primary skin fibroblasts (FEK4 cells), a type of cell derived from the dermal layer of human skin. These cells play a crucial role in maintaining skin integrity and function by producing extracellular matrix components such as collagen and elastin, which provide structural support and elasticity to the skin. FEK4 cells were seeded into a 96-well plate at a density of approximately 5,000 cells per well and incubated at 37°C with 5% CO_2 for 24 hours to allow for cell adhesion and growth, when the experiments were conducted. Gel prototypes containing different concentrations of elemental gold (24K gold powder) or microparticles of gold ranging from 200-700 nm were tested and prepared by diluting the gel in the culture media. The formulations were added to the wells in different ratios.

Formulation 0 was tested as a control of RSM gel without gold. The cells were exposed to the diluted formulations for 24 hours and their cytotoxic effects were assessed by MTT assay. The MTT reagent was added to each well and incubated for 3-4 hours, allowing the viable cells to convert the MTT into formazan crystals. After incubation, the media was carefully removed and the formazan crystals dissolved in a suitable solvent. The absorbance was measured at 570 nm using a microplate reader and the absorbance values of treated cells to those of control cells were compared to determine the cytotoxic effects of the formulations. This procedure effectively allows the cytotoxicity assessment of the various formulations on FEK4 cells.

2.3. Glutathione assay

To assess GSH levels, FEK4 cells treated with different RMS-GG formulations were seeded into appropriate culture plates to allow for adequate cell attachment and growth. After cells were confluent, they were treated with formulations added at different ratios into the culture media. In addition, a parallel set of formulation-treated cells received buthionine sulfoximine (BSO) at a concentration of 50 mM. BSO is a well-known inhibitor of gamma-glutamylcysteine synthase, the enzyme responsible for the first step in GSH synthesis. This makes it a useful control for assessing the impact of inhibited GSH synthesis. After 24 hours, a GSH assay was performed on all cells whether they were exposed to BSO or not. The assay involved calibrating the results using a GSH standard curve and the Bio-Rad protein assay (Bradford assay) to ensure accurate quantification of GSH levels. The data obtained were normalized to the protein content and expressed as nanomoles of GSH per milligram of protein. These values were then plotted as percentage changes in GSH levels relative to the untreated control group, which was arbitrarily set to a value of 1. The results were presented as the average of at least three independent experiments (n=3), with standard deviations shown as error bars to indicate variability and reliability of the data.

This protocol allows for a comprehensive analysis of GSH levels in FEK4 cells under different treatment conditions, providing insights into the cellular response to oxidative stress and the efficacy of potential therapeutic formulations. The use of BSO as an inhibitor helps to delineate the specific role of GSH synthesis in the observed results, making the data robust and informative.

2.4. Nrf2 activation assay

FEK4 cells were seeded into appropriate culture dishes to allow for attachment and growth. By day 2, the cells were treated with specific formulations of RSM-GG diluted at a ratio of 1:5 in the culture media. This treatment aimed to stimulate Nrf2 activity. On Day 3, the cells were fixed using a standard fixation protocol to preserve cellular structures. Immunocytochemistry was then performed using an Nrf2 antibody to detect the presence and localization of Nrf2, along with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear marker to stain the cell nuclei. Confocal microscopy was employed to capture detailed images of the cells, allowing for the visualization of Nrf2 within the cellular compartments. The images obtained were analyzed using ImageJ software to quantify the ratio of nuclear to cytoplasmic Nrf2 expression, providing insights into the extent of Nrf2 activation and its translocation to the nucleus.

2.5. NQO1 assay

The expression of NQO1 was investigated in cultured FEK4 cells. When proper attachment and growth were achieved, the cell monolayers were treated with specific formulations of RSM-GG diluted at a ratio of 1:5 in the culture media to stimulate NQO1 expression. By Day 3, the cells were fixed using a standard fixation protocol to preserve cellular structures and immunocytochemistry was performed using an antibody against NQO1 to detect its protein expression levels. Confocal microscopy was employed to capture detailed images of the cells and the fluorescence. The images were then analyzed using ImageJ software to determine the mean fluorescence intensity of NQO1 protein, providing quantitative data on the expression levels of NQO1 as an indicator of cellular response to the treatments.

3. RESULTS

3.1. Cytotoxicity-MTT assay

The safety of the different formulations of RSM-GG on FEK4 cells was assessed using the MTT viability assay. Data obtained from the MTT assays indicated that overnight treatments with the formulations, at the dilution ratios used, did not decrease the cell viability of the treated cells compared to the untreated control. This suggests that the formulations are non-toxic and safe for use under the experimental conditions, as the cell viability remained comparable to that of the untreated control cells. These findings confirm that the RSM-GG formulations do not adversely affect the viability of human primary skin fibroblasts, supporting their potential for further therapeutic applications (Figure 6).

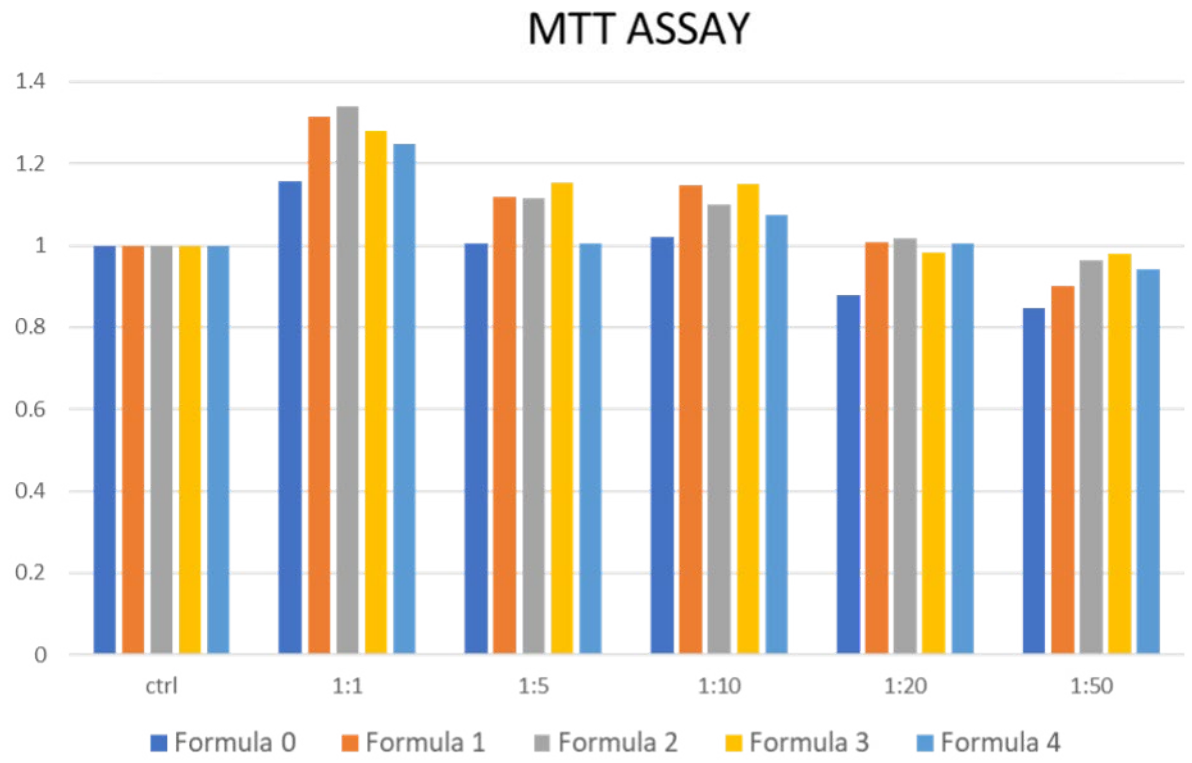


Figure 6. MTT assay conducted on human primary skin fibroblasts FEK4 cells for different ratios of each ASEA formulation. Data expressed as percentage cell viability compared to untreated control (Ctrl) set as 100%. Data presented as an average of at least 3 independent experiments (n=3) +/- standard deviations sown as error bars.
Formulation 0 - RSM + no gold added; Formulation 1 - RSM + 0.0033 mg/mL of gold; Formulation 2 - RSM + 0.0044 mg/mL of gold; Formulation 3 - RSM + 0.0055 mg/mL of gold; Formulation 4 - RSM + 0.0066 mg/mL of gold.

3.2. Glutathione/BSO assay

The treatment of FEK4 cells with different formulations of RSM-GG resulted in a significant increase in the intracellular levels of GSH after overnight incubation. Specifically, all formulations tested led to an elevation in GSH levels compared to untreated control cells, demonstrating the efficacy of RSM-GG in enhancing cellular antioxidant capacity. Conversely, treatment with 50 µM BSO alone markedly reduced the intracellular GSH levels by approximately 75% compared to the levels observed in untreated control fibroblasts, highlighting the potent GSH-depleting effect of BSO. Notably, in cells pre-treated with BSO, the various formulations of RSM-GG were able to restore the intracellular GSH levels, achieving up to 280% of the GSH levels (see

Formulation 4, Figure 7) compared to BSO-treated control fibroblasts. This restoration indicates that RSM-GG formulations possess the capability to counteract BSO-induced depletion of GSH, thereby supporting the maintenance of cellular redox balance. These results underscore the potential of RSM-GG formulations to mitigate oxidative stress by replenishing intracellular GSH levels in human primary skin fibroblasts, even under conditions of chemically induced GSH depletion.

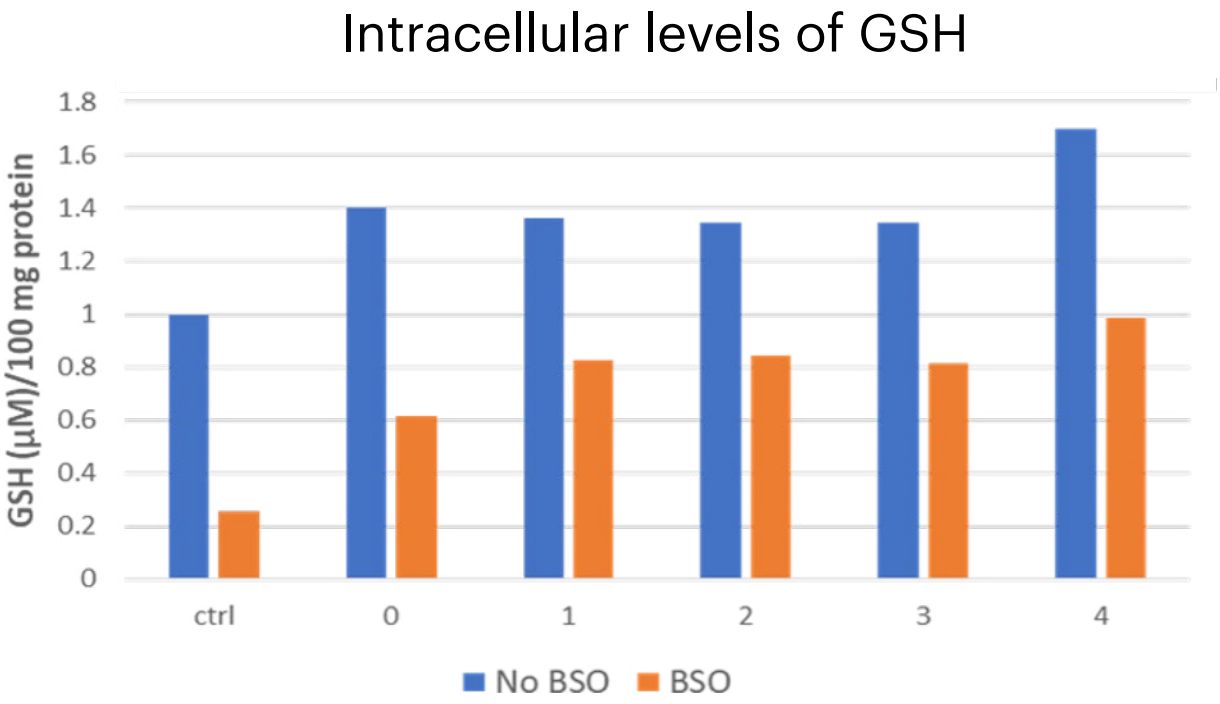


Figure 7. Overnight treatment of cells with all different formulations of RSM-GG with or without 50 µM BSO. Formulation 0 - RSM gel + no gold added; Formulation 1 - RSM gel + 0.0033 mg/mL of gold; Formulation 2 - RSM gel + 0.0044 mg/mL of gold; Formulation 3 - RSM gel + 0.0055 mg/mL of gold; Formulation 4 - RSM gel + 0.0066 mg/mL of gold.

3.3. Activation of Nrf2

The effect of RSM-GG formulations on Nrf2 translocation to the nucleus in FEK4 cells was evaluated using immunocytochemistry. Overnight treatment with RSM-GG formulations 1-6 demonstrated that all formulations promoted Nrf2 translocation to the nucleus, with an increase of up to 160% compared to the untreated control (Figures 8 and 9). These results are consistent with the observed abilities of these formulations to increase intracellular GSH levels, suggesting a correlation between Nrf2 activation and enhanced antioxidant capacity. The data indicates that the formulations not only boost GSH levels but also effectively activate the Nrf2 pathway, which is crucial for cellular defense mechanisms against oxidative stress.

The immunocytochemistry analysis revealed a significant increase in the nuclear localization of Nrf2 in cells treated with RSM-GG. This translocation is a key indicator of Nrf2 activation, as Nrf2 must move to the nucleus to initiate the transcription of antioxidant response element-driven genes. The observed increase in nuclear Nrf2 suggests that these formulations effectively trigger the Nrf2 pathway, enhancing the cell's ability to combat oxidative stress by upregulating the expression of detoxifying and antioxidant enzymes.

Furthermore, the correlation between Nrf2 translocation and increased intracellular GSH levels underscores the potential synergistic effects of these formulations. GSH, a major cellular antioxidant, plays a vital role in maintaining redox homeostasis and protecting cells from oxidative damage. The ability of RSM-GG formulations to elevate GSH levels and promote Nrf2 nuclear translocation highlights their dual role in bolstering the cell's antioxidant defenses.

Overall, these findings suggest that RSM-GG formulations are effective in activating the Nrf2 pathway and enhancing the antioxidant capacity of human primary skin fibroblasts. This dual action not only supports cellular health and resilience against oxidative stress but also underscores the potential use of these formulations in skin care and other applications where oxidative stress is a contributing factor.

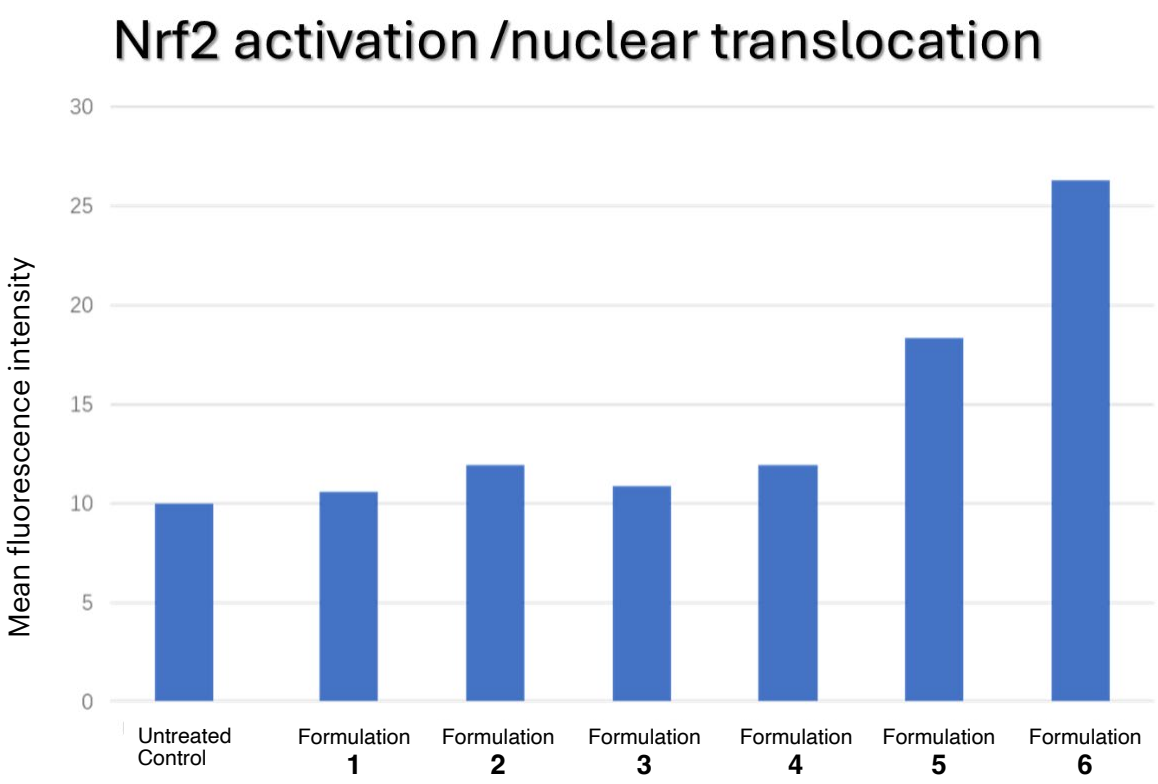


Figure 8. Overnight treatment of cells with formulations 0-6, revealed that all formulations promote Nrf2 translocation to the nucleus up to 160% (Formulation 6) compared to untreated control. Untreated control: no RSM or gold added; Formulation 1: RSM gel + no gold added; Formulation 2: RSM gel + 0.00025 mg/mL of gold; Formulation 3: RSM gel + 0.002 mg/mL of gold; Formulation 4: RSM gel + 0.004 mg/mL of gold; Formulation 5: RSM gel + 0.006 mg/mL of gold; Formulation 6: RSM gel + 0.008 mg/mL of gold.

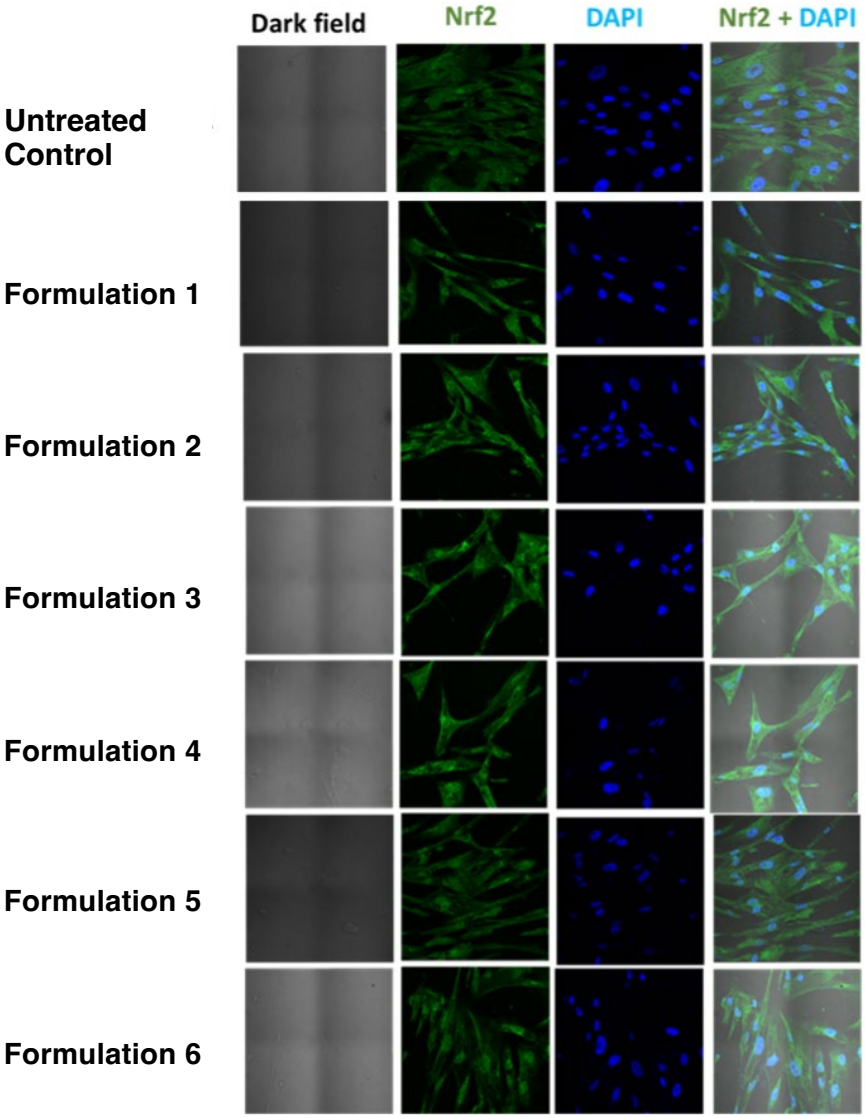


Figure 9. Immunocytochemical analysis of Nrf2 translocation in FEK4 Cells. FEK4 cells were treated overnight with various formulations and subsequently analyzed for Nrf2 translocation. Immunocytochemistry was performed using an Nrf2 antibody (green) to detect Nrf2 expression and DAPI (blue) as a nuclear marker. Confocal microscopy images were captured to visualize the localization of Nrf2 within the cells. ImageJ software was used to quantify the ratio of nuclear to cytoplasmic Nrf2 expression. Untreated control: no RSM or gold added;
Formulation 1: RSM gel + no gold added;
Formulation 2: RSM gel + 0.00025 mg/mL of gold;
Formulation 3: RSM gel + 0.002 mg/mL of gold; Formulation 4: RSM gel + 0.004 mg/mL of gold;
Formulation 5: RSM gel + 0.006 mg/mL of gold; Formulation 6: RSM gel + 0.008 mg/mL of gold.

3.4. NQO1 synthesis

Overnight treatment of FEK4 cells with formulations 1-4 revealed that all formulations promoted a significant increase in NAD(P)H dehydrogenase [quinone]-1 (NQO1) expression, with levels reaching up to 150% compared to untreated control (Figure 10). These findings are consistent with the observed activation of Nrf2 translocation to the nucleus, which subsequently promotes the activation of NQO1 expression in formulation-treated cells. Immunocytochemistry staining for NQO1 was performed, and confocal microscopy images were analyzed using ImageJ software to determine the mean fluorescence intensity of NQO1 protein. The increased NQO1 expression observed in the treated cells underscores the efficacy of the formulations in activating the Nrf2 pathway and enhancing the cellular antioxidant response. These results highlight the potential of RSM-GG formulations to bolster cellular defenses against oxidative stress through the upregulation of key antioxidant enzymes.

The immunocytochemistry analysis revealed a significant increase in NQO1 expression in cells treated with RSM-GG formulations 1-4 (Figure 10). This increase was quantified using confocal microscopy and ImageJ analysis, which showed that the mean fluorescence intensity of NQO1 protein was up to 150% higher in treated cells compared to untreated controls. The enhanced expression of NQO1 is indicative of the formulations' ability to activate the Nrf2 pathway, as NQO1 is a downstream target gene of Nrf2. The translocation of Nrf2 to the nucleus, observed in previous experiments, likely facilitated the transcriptional activation of NQO1, thereby increasing its protein levels.

These findings suggest that the formulations not only enhance the antioxidant capacity of the cells by increasing GSH levels but also activate the Nrf2 pathway, leading to the upregulation of NQO1. This dual mechanism of action shows the therapeutic potential of RSM-GG formulations in protecting cells from oxidative stress. The ability to increase NQO1 expression further supports the role of these formulations in enhancing cellular resilience and defense mechanisms. Overall, the data provide strong evidence for the efficacy of RSM-GG formulations in promoting antioxidant responses and protecting human primary skin fibroblasts from oxidative damage.

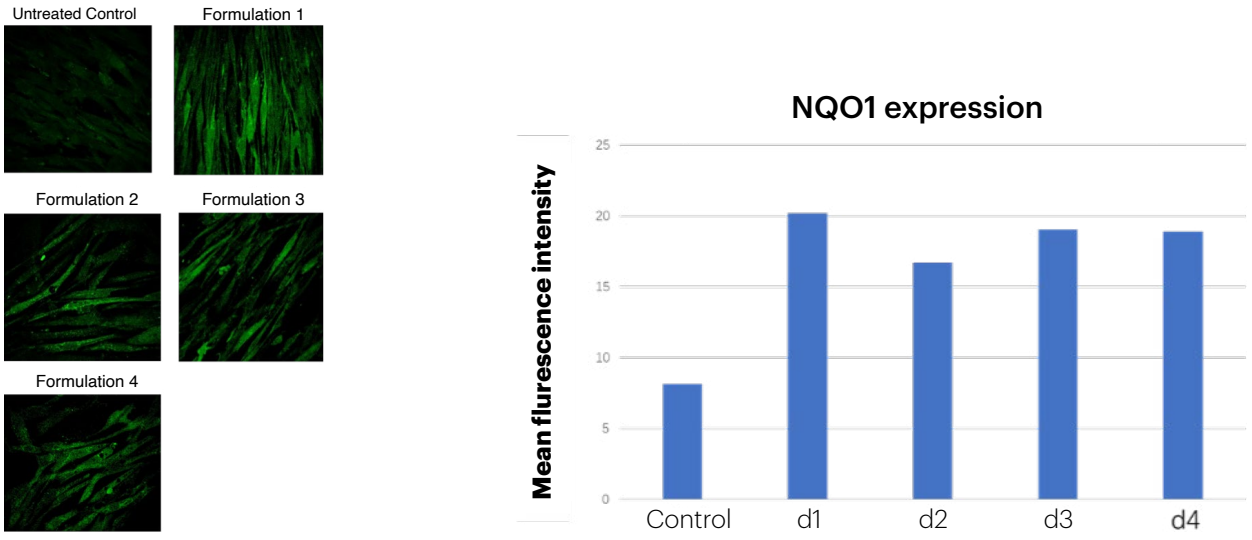


Figure 10. Overnight treatment of cells with RSM-GG formulations 1-4, shows that all formulations promote an increase in NQO1 expression to up to 150% compared to untreated control. Control - no formulation added; Formulation 1 - RSM + 0.0033 mg/mL of gold; Formulation 2 - RSM + 0.0044 mg/mL of gold; Formulation 3 - RSM + 0.0055 mg/mL of gold; Formulation 4 - RSM + 0.0066 mg/mL of gold.

4. CONCLUSIONS

The evaluation of RSM-GG formulations on FEK4 cells demonstrated their safety and efficacy through various assays, as follows.

- Overnight treatments with the tested formulations, at specified dilution ratios, did not decrease cell viability compared to untreated controls. This indicates that the formulations are non-toxic and safe for use under experimental conditions.
- Treatment with RSM-GG formulations significantly increased intracellular GSH levels, enhancing cellular antioxidant capacity.
- RSM-GG formulations restored GSH levels by up to 280% in BSO-treated cells, counteracting chemically induced GSH depletion and maintaining cellular redox balance.
- RSM-GG formulations promoted an increase in the Nrf2 translocation to the nucleus by up to 70% compared to untreated controls. Activation of the Nrf2 pathway is crucial for cellular defense against oxidative stress, leading to the upregulation of detoxifying and antioxidant enzymes like GSH and NQO1.
- Correlation between Nrf2 translocation and increased GSH levels underscores the synergistic effects of these formulations in bolstering the cell's antioxidant defenses.
- RSM-GG formulations significantly increased the expression of NQO1, with levels reaching up to 150% compared to untreated controls.
- This increase in NQO1 expression supports the activation of the Nrf2 pathway by RSM-GG formulations, enhancing the cellular antioxidant response and resilience against oxidative stress.

5. DISCUSSION

This study demonstrates that RSM-GG formulations are both safe and effective in enhancing the antioxidant capacity of human primary skin fibroblasts, supporting their non-toxic nature and potential for topical use.

The increase in Nrf2 translocation to the nucleus indicates that these formulations effectively activate the Nrf2 pathway, a critical transcription factor that regulates the expression of various antioxidant and detoxifying enzymes. Its activation leads to the upregulation of genes involved in the cellular defense against oxidative stress.

Additionally, RSM-GG formulations significantly boost intracellular glutathione levels, which are crucial for maintaining cellular redox balance. The correlation between Nrf2 activation and the increased GSH levels highlights the synergistic effects of these formulations in enhancing the cell's antioxidant defenses. Moreover, this ability to restore GSH levels in BSO-treated cells underscores their potential to counteract oxidative stress and maintain cellular homeostasis, thereby enhancing cellular resilience and recovery.

Having a topical gel that restores GSH levels is crucial because it directly addresses oxidative stress caused by free radicals (32), which are known to be a major factor in aging (33). GSH is a powerful antioxidant that helps neutralize harmful free radicals, thereby decreasing oxidative stress and maintaining the redox balance within cells (34). Depletion of GSH simulates the oxidative damage that occurs in compromised cells, contributing considerably to premature aging, inflammation, and various other disorders. By restoring GSH levels, a topical gel can help maintain cellular homeostasis, ensuring that cells function optimally even under stress. This not only enhances recovery at cellular level, but also helps in protecting cells from environmental aggressors like UV radiation and pollution but also supports natural repairing processes.

Further insights were gained from the Nrf2 activation and NQO1 synthesis assays. The increase in Nrf2 translocation to the nucleus indicates that RSM-GG formulations activate the Nrf2 pathway, leading to the upregulation of genes involved in antioxidant defense. This study demonstrates a significant increase in NQO1 expression, highlighting the role of NQO1 as a key

enzyme in the detoxification of quinones and in providing protection against oxidative damage. Unlike glutathione, which acts systemically, NQO1 functions as a more targeted antioxidant (35, 36). By enhancing cellular resilience through these mechanisms, the formulations support the cell's ability to recover and maintain homeostasis. Additionally, it can particularly benefit specific tissues, including joint tissues, by helping mitigate oxidative stress and supporting joint health and recovery (37).

The NQO1 synthesis assay results reinforced the findings from the Nrf2 activation assay. The significant increase in NQO1 expression in cells treated with RSM-GG formulations suggests that these formulations not only activate Nrf2 but also promote the expression of its downstream target genes. It plays a crucial role in specific tissues, like joint tissues, where it helps mitigate oxidative stress and, consequently, supports joint health and recovery. The upregulation of NQO1 further supports the role of RSM-GG formulations in bolstering the cellular antioxidant response, particularly in areas where targeted antioxidant action is needed.

In summary, these results suggest that RSM-GG formulations have a dual mechanism of action: they increase intracellular GSH levels, providing antioxidant protection, and activate the Nrf2 pathway, leading to the upregulation of targeted antioxidants like NQO1. This dual action enhances the antioxidant capacity of human primary skin fibroblasts, providing a robust defense against oxidative stress. The non-toxic nature of these formulations, combined with their ability to enhance both overall and targeted antioxidant defenses, underscores their potential in skin care and other applications where oxidative stress is a contributing factor.

6. LITERATURE

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