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Squalene is a potential hypothyroidism modulator: a study of its effect on the Keap1-Nrf2-ARE signaling pathway in potassium dichromate induced hypothyroidism model in rats.

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Abstract

Background: Assessing the pharmacological effect of squalene alone and in combination with vitamin E, and their antioxidant activity against the hypothyroidism induced by potassium dichromate, via mechanistically studying Keap1-Nrf2-ARE pathway and their cyto-protective effects. Materials and methods: Seventy male albino rats were divided into seven groups (n=10), received the following orally on daily basis for 4 weeks, group I as negative control group receiving only saline, group II received squalene only 0.4 ml/day/rat, group III received vitamin E only (250 mg/kg), group IV was the positive control group receiving potassium dichromate only (2.5 mg/kg). Group V received potassium dichromate and squalene, group VI received potassium dichromate and vitamin E, while the last group received a combination of all three together. Results: groups treated with squalene and/or vitamin E showed a great help in modulating the damaging effect of potassium dichromate on thyroid gland, this as proved by elevation of Nrf2, T3, T4, reduced GSH, as well as reduction of KEAP 1, TNF-α, IL-6, TSH, MDA and NO when compared to positive control group.

Conclusion: Squalene and vitamin E help in reversing the condition of hypothyroidism and managing its progression by directly interacting with the Keap1-Nrf2-ARE pathway in the thyroid gland.

Keywords: Hypothyroidism, Keap1-Nrf2-ARE pathway, squalene, vitamin E, antioxidants, ROS, OS

1. Introduction

Several studies have monitored the effect of antioxidants on hypothyroidism but to the best of our knowledge it has not been reported before linking squalene and vitamin E with it and discussing their effects on the Nrf2-Keap1 pathway. Hypothyroidism is considered one of the most common and critical endocrine disorders mainly caused by alteration in the thyroid gland, leading to progression in thyroid hormones produced (1).

People are subjected to various chemicals that have a negative impact on our bodies. One of these is chromium (Cr), which is a heavy metal with a notable toxic effect, it is used in many chemical industries, and it specially insults the thyroid gland and may result in hypothyroidism. This insult is correlated to its potent oxidizing effect showing significant affinity when reduced to trivalent Cr ($cr3^+$) affecting various body organs (2) as kidney (3), brain (4), lung (5) and liver (6).

Cr is detected in different oxidation states, the most common are trivalent ($cr3^+$) and hexavalent cr forms. Hexavalent cr is rapidly up-taken by the cells and quickly reduced, this explains why it is more toxic than trivalent cr form (3). Cr ties up with oxygen resulting in a strong oxidizing agent, toxicity happens mainly due to production of enormous amounts of free radicals and ROS (ROS), resulting in a cascade of damaging events, inflammation and tissue injury. ROS and oxidative stress (OS) is then the main cause of lipid peroxidation, DNA damage which significantly may be the cause of hypothyroidism (17).

The kelch-like ECH associated protein 1/nuclear factor erythroid 2-related factor 2 (Keap-1/Nrf-2) antioxidant protective system reacts to reduce OS (8). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a significant transcription factor that unswervingly

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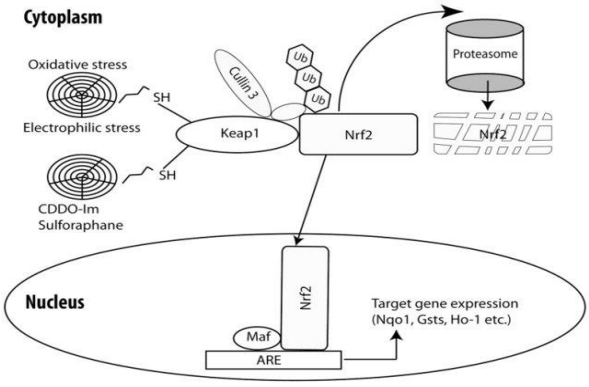
affects antioxidant proteins expression including antioxidant enzymes, including hemeoxygenase-1, thioredoxin, peroxiredoxins (Prxs), and Sulfiredoxin (Srx). Several studies have shown that OS induces the expression of the cysteine uptake transporter and heme oxygenase-1 through the activation of Nrf-2. These proteins are considered as backbone in the management of oxidative stress and balancing its cellular effect (9).

Nrf2 is released from the Nrf2-Keap1 in normal conditions, Nrf2-Keap1 is the inhibitory complex vulnerable to proteasomes in the cytoplasm. During OS and ROS, NRF-2 is translocated to the nucleus, leaving Keap-1 behind, to stimulate ARE (antioxidant responsive elements) (10).



Keap1/Nrf2/ARE pathway represents one of the leading cellular protective mechanisms against OS and xenobiotic damage. The Keap1/Nrf2/ARE pathway significantly contributes in health strength against inflammatory diseases, neurodegenerative diseases, Parkinson's disease, stroke, chronic kidney disease, atherosclerosis, diabetes, cardiovascular diseases, and rheumatoid arthritis (11).

Squalene (SQ) is a natural triterpene widespread in nature. It is a product of metabolism produced in the process of sterol biosynthetic pathway. Many previous studies proved its scavenging activity towards free radicals, meanwhile some studies have focused on its effect in OS (12). It is well known that antioxidants can scavenge the free radicals ROS and abrupt more deterioration in many medical conditions, that attracted our attention to choose squalene as an antioxidant found as a triterpene present in olive oil, pumpkin seed oil as well as shark oil as isoprenoid molecule. It was proved previously to have anti-lipidemic, antioxidant as well as membrane stabilizing properties (13) and its combination with another well-known potent antioxidant as vitamin E was to assess whether it will synergistically aid squalene or not in preventing the deterioration caused by free radicals and interfering with the main Keap1-Nrf2-ARE pathway in the thyroid gland.

2. Materials and methods

2.1 Animals

Adult male albino Wister rats, were used in the current study, with average weights of 120-140 g, and were bought from the National Research Centre (NRC; Cairo, Egypt). Guidelines of the animal care and use committee of the NRC were followed. Throughout the experimental period, animals had free access to food and water as well as keeping them in a quiet area. Guiding principles for animal experimentation were followed as enunciated by the US guidelines (NIH publication #85-23, revised in 1985) and with the Instructional Animal Care and Use Committee (IACUC).

2.2 Drugs and kits

Squalene (P97% by GC) was purchased from Sigma Chemicals Company, St. Louis, MO, USA. Potassium dichromate was bought from El-Nasr pharmaceutical chemicals. Vitamin E was purchased from Sigma Aldrich, Nitric oxide (NO), was bought from Biodiagnostic, Egypt. Keap1-Nrf2 ELISA kits were purchased from Sinogeneclon Biotech Co., Ltd, China, While IL-6 was purchased from NOVA, Beijing, China. TNF-α ELISA kit was bought from SunRed China. T4 and TSH were obtained from Elabscience, USA, While T3 was purchased by Aviva Systems Biology, San Diego, USA. All other chemicals used were of the highest purity and analytical grade.

2.3 Experimental design

Seventy male albino rats were used and divided into seven groups (n=10), group I was maintained as the negative control group receiving only saline, group II received squalene only (0.4 ml/day/rat) (14, 15) orally on daily basis for 4 weeks, and group III received vitamin E only (250 mg/kg) orally on daily basis for 4 weeks (16), group IV was maintained as a positive control group receiving potassium dichromate only (2.5 mg/kg) orally for 4 weeks (17). Group V received potassium dichromate and squalene, group VI received potassium dichromate and vitamin E, while the last group received a combination of all three together of potassium dichromate, squalene and vitamin E with the same mentioned doses.

At finalizing the experiment, blood samples were collected via the retro-orbital plexus of each anesthetized fasting rat for 12 hr, this was performed by the aid of a glass capillary tube. Blood samples were centrifuged using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 rpm for 15 min. The serum was kept at -20 °C until analyzed. Targeted estimation of Keap 1 and Nrf2 expression was performed. MDA and reduced GSH were quantified in the serum samples as well as estimation of inflammatory mediator TNF- α and IL-6, NO. TSH and thyroid hormones T3 and T4 were estimated in serum as well.

2.4 Statistical analysis

Data are expressed as mean ± S.E. of values obtained from (n=10) rats. Statistical analysis of data was performed with graphpad Prism 4.0 (graphpad software, USA). Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Statistical significance was considered when probability values (P) were less than 0.05.

3. Results

3.1 Effect of treatment on serum TSH, T3 and T4.

Serum level of TSH was elevated in potassium dichromate group by 220%, as compared to normal group, while the treatment with squalene, vitamin E and their combination showed a reduction of TSH by 16.5% 41.9% and 54.1%, respectively when compared to potassium dichromate positive control group rats. While potassium dichromate injection showed a reduction of T3, T4, serum levels by 26.6%, and 47%, respectively as compared to the normal group. While squalene and vitamin E and their combination administration showed an elevation of T3 by 2.5.%, 15.3% and 31.6%, T4 by 16.1%, 38.9% and 61.2%, respectively as compared to potassium dichromate positive control group rats. **(Table 1)**

Table (1): Effect of treatment on serum (A) TSH, (B) T3 and (C) T4. T3 and (C) T4.

Group	T3 (pmol/L)	T4 (pg/ml)	TSH (ng/ml)
Normal	0.4785 ± 0.0007	3.55 ± 0.067	1.155 ± 0.020
NS	0.4625 ± 0.007	3.5 ± 0.204	1.536 ± 0.049
NE	0.4516 ± 0.008	3.8 ± 0.044	1.55 ± 0.156
Pos	0.351 ± 0.12 *	1.885 ± 0.006 *	3.7 ± 0.044 *
S	0.36 ± 0.0045 *#	2.19 ± 0.0044 *#	3.09 ± 0.026 *#
E	0.4054 ± 0.0025 #	2.62 ± 0.0089 #	2.15 ± 0.022 #
SE	0.4624 ± 0.0011 #	3.04 ± 0.017 #	1.7 ± 0.044 #

Data are presented as mean ± SE of 10 rats per group, using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$. Where, * vs Normal; # vs pos.

3.2 Effect of treatment on serum reduced GSH, NO and MDA

Potassium dichromate injection showed a reduction in reduced GSH serum levels by 42.9%, as compared to normal group, while squalene and vitamin E and their combination administration showed an elevation of reduced GSH serum levels by 20.5%, 35.4% and 63% respectively as compared to potassium dichromate positive control group rats. Potassium dichromate injection showed an elevation of NO and MDA by 35.6% and 25.29% respectively as compared to the normal group. While squalene and vitamin E and their combination administration showed a reduction of NO serum level by 15.8%, 25.84.% and 27.2% and MDA serum level by 35.27%, 51.75% and 50.87% respectively as compared to potassium dichromate positive control group rats. **(Figure 1)**

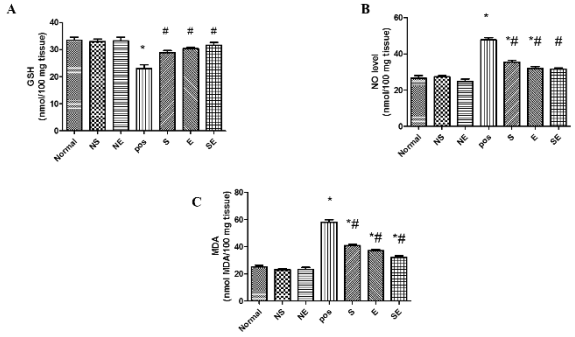


Figure (1): Effect of treatment on serum (A) reduced GSH, (B) NO and (C) MDA.

Data are presented as mean ± SE of 10 rats per group, using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$. Where, * vs Normal; # vs pos.

3.2 Effect of treatment on serum TNF-α and IL6.

Potassium dichromate injection showed an elevation of TNF-α and IL6, by 110.16%, 61.2 %, respectively as compared to the normal group. While squalene and vitamin E and their combination administration showed a reduction of TNF-α serum level by 18.55%, 37.5% and 47.18%, IL6 serum level by 8.81%, 19.2% and 27.2% respectively as compared to potassium dichromate positive control group rats. **(Figure 2)**

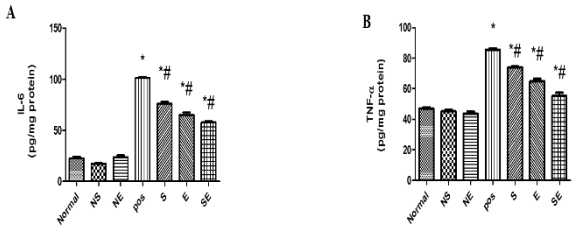


Figure (2): Effect of treatment on serum (A) TNF-α and (B) IL-6.

Data are presented as mean ± SE of 10 rats per group, using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$. Where, * vs Normal; # vs pos.

3.3 Effect of treatment on serum expression of KEAP1/Nrf2.

Serum level of keap1 was elevated in potassium dichromate group by 182.4%, as compared to normal group, while the treatment with squalene, vitamin E and their combination showed a reduction of keap 1 by 4.1.% 18.7.% and 39%, respectively when compared to potassium dichromate positive control group rats. Meanwhile Serum level of Nrf2 was reduced in potassium dichromate group by 51%, as compared to normal group, while the treatment with squalene, vitamin E and their combination showed an elevation of Nrf2 by 173.9% 219.2% and 657.7%, respectively

when compared to potassium dichromate positive control group rats. **(Figure 3)**

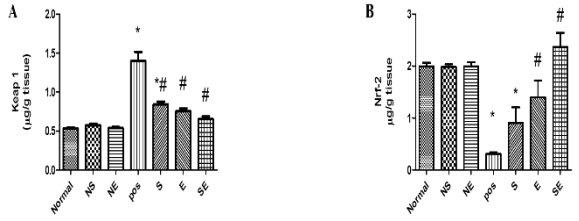


Figure (3): Effect of treatment on serum (A) Keap1 and (B) Nrf2.

Data are presented as mean ± SE of 10 rats per group, using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$. Where, * vs Normal; # vs pos.

4. Discussion

Potassium dichromate is a hexavalent form of Cr used for induction of hypothyroidism. Cr reduced intermediates are asserted to react with hydrogen peroxide forming hydroxyl radicals a potent well known free radical (18) that excellently clarifies why potent antioxidants used in this study (squalene and vitamin E) were able to reduce the incredible increase in inflammatory markers, ROS and OS markers.

Toxicity due to toxic substances induces generation of huge amounts of free radical, which results in lipid peroxidation, OS and drastic injury on the cellular level. Squalene proved its stronger scavenging effect of hydroxyl free radicals even more than endogenous reduced glutathione (GSH). It was previously proved that antioxidants have a broad spectrum positive impact on our bodies (19). Our results explored that treating the groups receiving potassium dichromate with squalene and/ or vitamin E showed elevation in the level of reduced GSH as well as reduction in the elevated level of MDA when compared to the positive control group, which was in harmony to the results of a previous study (20).

Meanwhile it was established by previous studies that vitamin E has the ability to safeguard against known induced ROS hepatotoxicity, genotoxicity, nephrotoxicity which estimated MDA levels as an OS marker and ascertained that it was elevated upon intoxication with potassium dichromate (21). Previous studies discovered a potent scavenging effect of squalene in comparison to vitamin E in aged population (22). This was totally in harmony with our findings that showed better results in groups receiving squalene more than those receiving vitamin E, while the concurrent use showed best results.

OS is enmeshed in both hypo- and hyper-thyroid disorders. Under conditions of low OS, Nrf-2 interacts with Keap-1/Nrf-2, in the cytoplasm, vulnerable to decadence in proteasome. But if the cellular level of ROS was elevated, ROS is capable of oxidizing redox-sensitive cysteine residues on Keap-1 and generating the decadence of the Keap1/Nrf-2 complex. Nrf-2 is then capable of translocating to the nucleus and binding to antioxidant responsive elements (ARE) of many antioxidant genes and regulating their expression (23). Our study proved that during OS (potassium dichromate model group) the KEAP1-NRF2 complex was disconnected. Nrf-2 has a role in the expression of antioxidant enzymes in other tissues (24).

Keap1/Nrf2 signals in the thyroid gland against OS, and many studies have proved that the antioxidant response pathway centered on Nrf2 is the defensive mechanism. Nrf2 is a conserved leucine zipper protein that acts as a key player in tissue proteostasis via upregulation of the transcription of antioxidant defense genes, and downregulating the transcription of pro-inflammatory cytokines (25). In normal conditions, Nrf2 is attached to its cytoplasmic inhibitory complex formed Keap1 and Cullin 3 (Cul3), wherein Keap1 targets Nrf2 for polyubiquitination by Cul3 leading to subsequent decaying by the proteasome. Under Os conditions, certain redox reactive cysteines of Keap1 become oxidized, thereby vanishing its capability to target Nrf2 for polyubiquitination, and degradation (26). Nrf2 is accordingly stabilized and accumulates in the nucleus, where it binds to DNA sequences called antioxidant Response Element (AREs) that are located in the promoters, and enhancers of its numerous target genes (27).

The current results demonstrated that administration of squalene and/ or vitamin E has a significant effect as it reduced keap-1 and elevated Nrf2 in potassium dichromate treated groups, best results were achieved by the combination, this was in parallel to a previous valuable study that explored the effect of resveratrol on potassium dichromate model and their effect on this pathway as well (10). Therefore it was speculated that this pathway is engaged in a cyto-protective defensive mechanism system. Similarly Padiya et al 2014 proved that garlic as an antioxidant agent enhances and improves keap1/Nrf2 pathway and modulates the effect of ROS (28). Linking all of the up-mentioned results together reveals that Keap1/Nrf2/ARE antioxidant system provides a cyto-protective effect against ROS produced by potassium dichromate, and that squalene and/or vitamin E are apprehended as strong antioxidants that improves the cyto-protective effect.

By the same token, the pro-inflammatory process was shown to be triggered by the significant elevation of TNF-α, NO and IL-6 upon the use of potassium dichromate and the upshot of ROS generation. Meanwhile, squalene and vitamin E treated groups depicted a significant improvement in all parameters indicating their potent anti-inflammatory. In normal conditions, our bodies produce ROS, are necessary for normal thyroid cell proliferation and the leading hormones are synthesized and produced via thyroid follicular cells, these include triiodothyronine (T3),

and thyroxine (T4) (29, 30). However, an uncontrolled huge amount of ROS triggers OS, which is a playmaker in the pathogenesis of a widespread diseases due to inflammation (31).

Hexavalent Cr can stimulate several pathways, elevating cytokine production specially TNF-α. Cytokines play a critical role by activating cell apoptosis, and an inflammatory cascade of events, and TNF-α and IL-6 emerge primitively in circulating blood (32). TNF-α was positively correlated with interleukins which increase in its expression TNF-α as a result of increased OS. Our results were in harmony with a previous study that showed that TNF-α and IL-6 were elevated upon intoxication with potassium dichromate and were reduced upon using a preventive antioxidant (32).

Our study also documented elevation in the positive control group NO level and reduction upon combination of treatment, which was in harmony with a previous study that proved NO OS activates inducible nitric oxide (iNOS) resulting in excessive production of NO and peroxynitrite (33).

Our study proved that potassium dichromate has a toxic effect on the thyroid gland due to creating a significant oxidative damage and production of ROS, this was proved by a notable decrease in free T3 and T4 levels thyroid tissues were insulted and hypo-functioning was detected in potassium dichromate intoxicated group showing histological changes revealing a significant decrease in T3, T4 and increase in TSH, this best explains our similar results since the damage caused to thyroid gland resulted in reduced blood level of circulation free thyroid hormones (T3 and T4) while TSH is elevated, since the later stimulates the follicles to synthesize and secrete more hormones into the circulation in an attempt to compensate for decreased thyroid hormones (34). Similar findings were reported by Aboul-fotouh et al, 2018, who attributed impairment of thyroid hormones by follicular cells to increase OS and ROS that resulted during reduction of potassium dichromate to trivalent form. Other research (34) attributes the fall in T3 and T4 to active combination of Cr with globulins. This in turn hindered the process of thyroglobulin proteolysis.

Antioxidants guard cells as well as organs from damaging effects of free radicals. Ibrahim et al., 2018 proved that rats receiving Vitamin C and/or ginseng in potassium dichromate model rats caused a marked reduction in TSH and marked elevation in T3, T4, FT3 and FT4 concentrations in comparison with potassium dichromate intoxicated group, it also showed improvement in the thyroid gland structure (7). TSH levels were back to normal and T4 and T3 levels were improved. (35). Moreover, vitamin c caused a marked elevation in the level of the T3 and T4 and a marked reduction in the TSH concentration compared to potassium dichromate intoxicated rats (36), their results strengthen our results which showed elevation in T3 and T4 hormones and reduction in TSH upon the use of squalene and/or vitamin E as strong antioxidants administered concurrently with potassium dichromate.

5. Conclusion

Our results elucidates that the treatment of squalene and vitamin E alone or in combination showed promising anti-inflammatory properties as well as antioxidant properties in vivo, and best results were usually achieved in the combination between both of them, since they were able to correct and reverse the induced hypothyroidism explaining the mechanistic pathway and linking Keap1-Nrf2-ARE pathway.

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