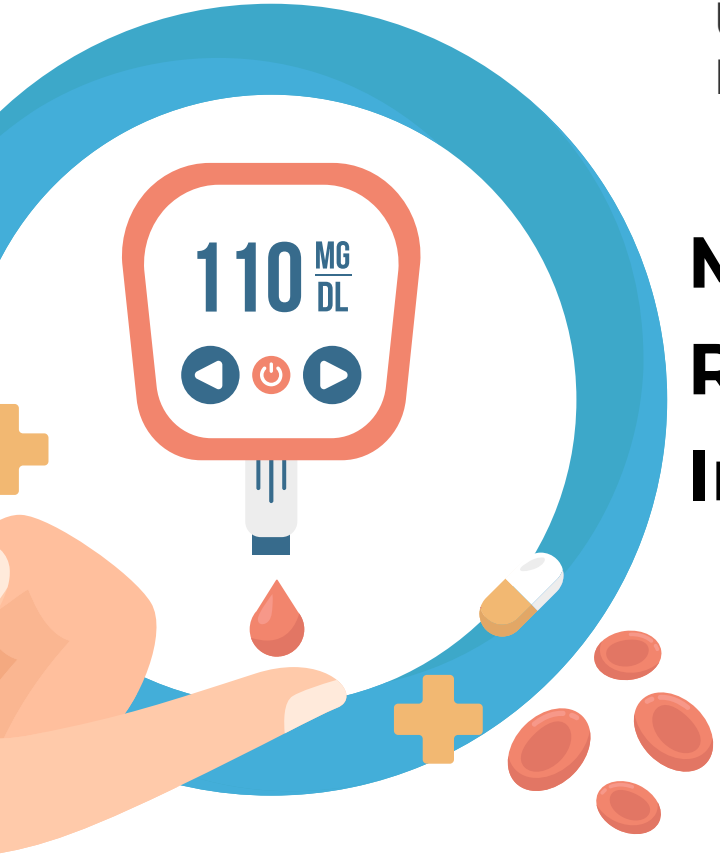

Under supervision of Dr. Reem Arafa
BMS 204 - Spring 2024

Muscle Oxidative Stress Plays a Role in Hyperthyroidism-Linked Insulin Resistance

A Comparative Study

Keywords: Hyperthyroidism, Insulin resistance, Diabetes, ROS, Vitamin E, Slc2a1, Slc2a4

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BACKGROUND

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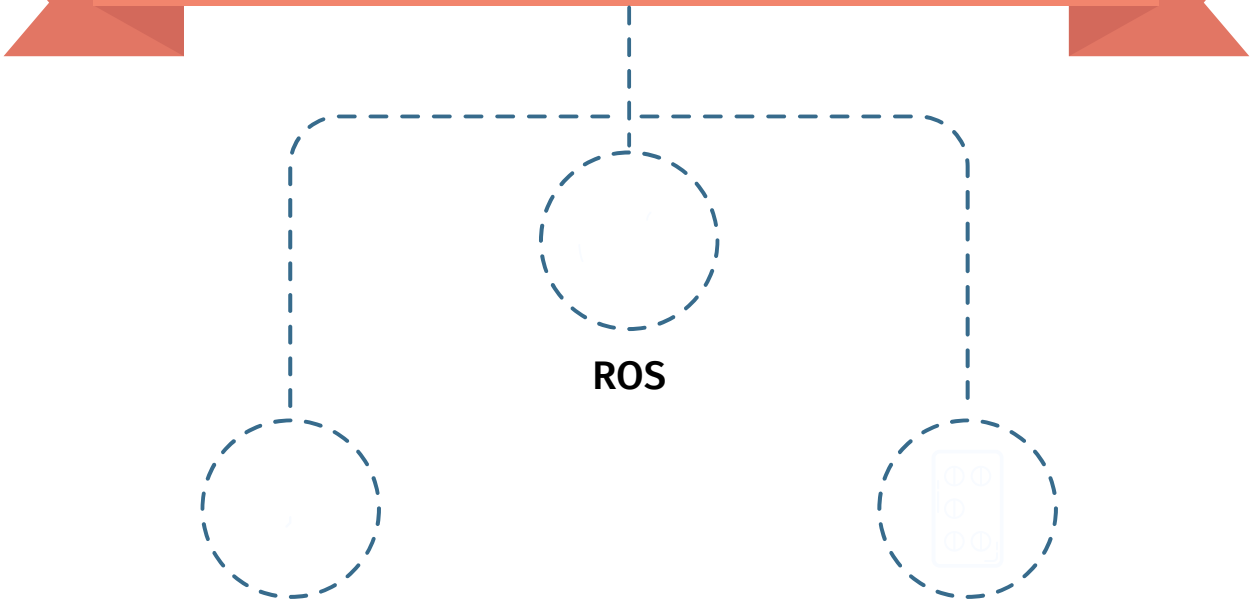
THE AIM

Insulin resistance, a common dysfunction linked to oxidative stress, is linked to type 2 diabetes mellitus, a metabolic disease causing 1.5 million deaths annually [1]. This study aims to:

- investigate the mechanisms of insulin resistance in skeletal muscle under hyperthyroid conditions, focusing on oxidative stress and vitamin E supplementation's potential benefits in reducing oxidative damage and improving insulin sensitivity.

- examine the impact of hyperthyroidism and vitamin E supplementation on gene expression associated with glucose transporters (Slc2a1; Slc2a4), lipid homeostasis (Pparg; Ppara, Cd36), and inflammatory factors (Il1b) in skeletal muscle.

3 MAIN PILLARS



Reactive Oxygen Species (ROS)

ROS are reactive oxygen-containing molecules involved in physiological and pathological processes [2]. They are divided into free radicals and non-radical species, and are produced as byproducts of cellular metabolism, mainly from mitochondrial electron transport chains and oxidation-reduction reactions.

- ➔ **Superoxide Anion (O2•-)**
- ➔ **Hydrogen Peroxide (H2O2)**

Generation of Superoxide Anion ($O_2^{\bullet -}$)

- Various oxidases facilitate this coupling reaction, including NADPH oxidase, uncoupled NOS, xanthine oxidase (XO), and complexes I/III/IV of mitochondria [3].

- The initial step involves the generation of superoxide ($O_2^{\bullet -}$) through mono-electronic reduction of O_2, leading to the generation of superoxide anion [4].

- This process results in the creation of a charged ionic species with a single unpaired electron and a net negative charge of −1.

Generation of Hydrogen Peroxide (H_2O_2)

- Superoxide dismutase (SOD) in mitochondria is an antioxidant protein that facilitates the rapid conversion of superoxide ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) [3].

- Certain oxidases with dismutase activity, like DUOX1/2 and NOX4, can directly convert O_2 to H_2O_2.

- Peroxisomes produce H_2O_2 as a byproduct of fatty acid oxidation and lipid metabolism.

The Redox Equilibrium (Antioxidant Defense System)

- ROS are vital for cell defense, but excessive levels can cause oxidative stress, damaging DNA, proteins, and lipids, leading to cell dysfunction and death [2].

- Enzymes like SOD, catalase, and glutathione peroxidase, along with non-enzymatic antioxidants like glutathione, vitamin C, and vitamin E, work together to safeguard cells from ROS-induced damage [3].

- Methionine oxidation by ROS converts methionine residues to methionine sulfoxides, but methionine sulfoxide reductase prevents this process, preserving protein stability and integrity.

Insulin resistance (IR) as a dysfunction of oxidative stress occurs and hyperthyroidism

- Evidence suggests a connection between insulin resistance and abnormal cellular ROS concentrations, particularly H_2O_2 and $O_2^{\bullet-}$ [3-5].

- Research by Zhang et al. [3] indicates that increased intracellular H_2O_2 concentration contributes to insulin resistance in obese mouse models.

- Catalase deficiency, highlighted in a review by Klaudia et al. [4], is linked to insulin resistance and other metabolic disorders.

- T3 administration reduces GLUT4 expression in hyperthyroid rats' plasma membrane, promoting NADPH oxidase expression and increasing NADPH-dependent H_2O_2 synthesis [6,7].

ROS and PI3K-Akt Signaling Pathway

- The intracellular pathway PI3K/AKT governs critical cellular processes like drug resistance, apoptosis, cell cycle regulation, and protein synthesis in response to growth factors, cytokines, and hormones.

- Insulin binding activates PI3K and PDC, facilitating the translocation of GLUT4 to the plasma membrane through tyrosine phosphorylation of the receptor substrate.

- Insulin binding to PI3K activates NOX, promoting superoxide anion formation and H2O2 conversion. NOX enhances glucose absorption by blocking PTPs and promoting IRS tyrosine phosphorylation [5,6].

- JNK (c-Jun NH2-terminal kinase), activated by mitochondrial H2O2 and NOX, triggers angiotensin II (AngII) through Ang II type 1 receptor AT1R, leading to IRS serine phosphorylation, reducing glucose uptake [6].

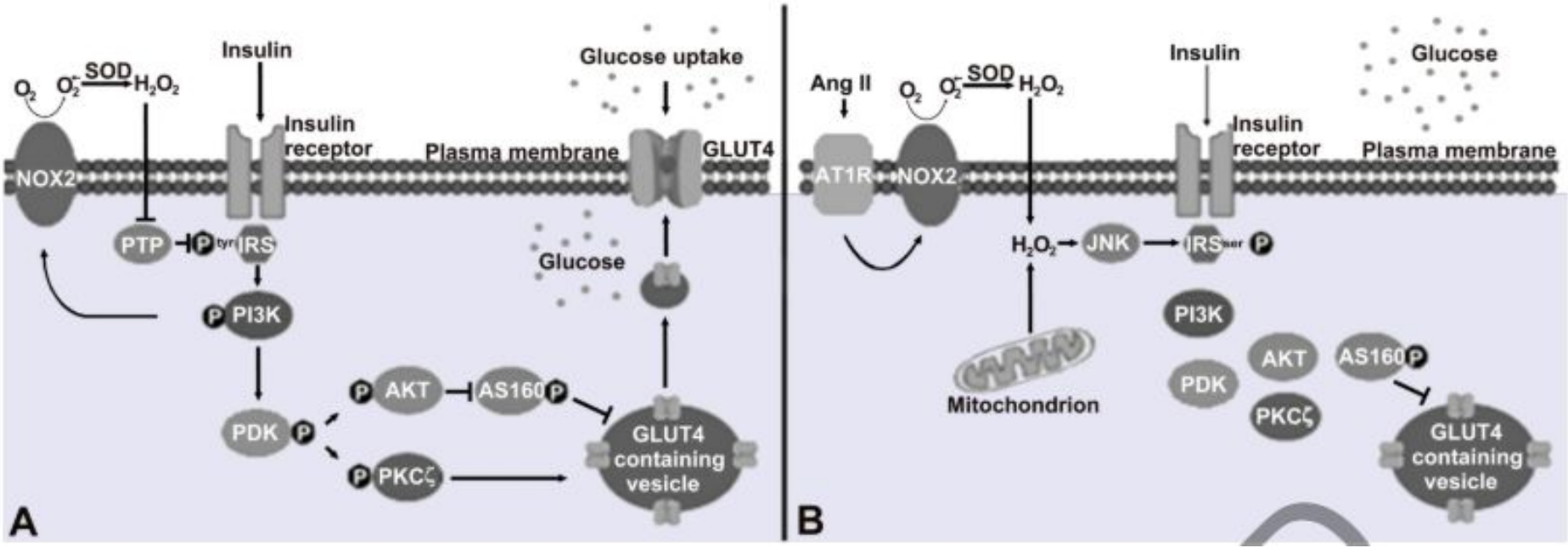


Fig.1: (A) Skeletal muscle representation of insulin-stimulated glucose uptake (B) and IR [6].

Vitamin E

Also known as Alpha-Tocopherol (C29H50O2), is a vitamin whose structured as:

A chromanol ring with a hydroxyl group that donates hydrogen atom to scavenge free radicals and a hydrophobic side chain that allows Vitamin E to insert itself into the cell membrane and scavenge lipid-based free radicals (8)

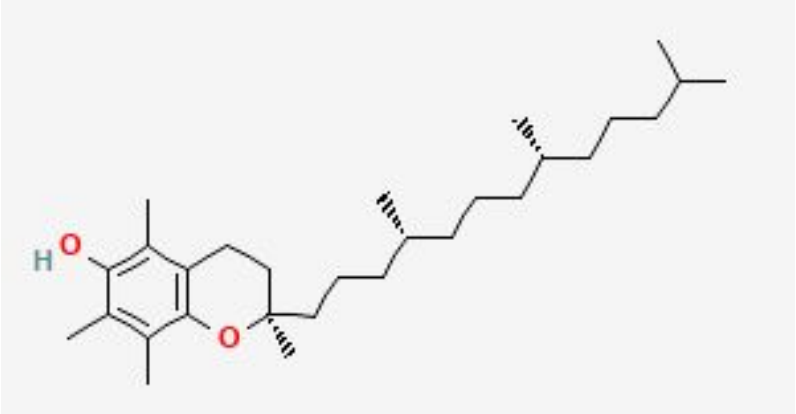


Fig.2: Alpha- Tocopherol Chemical Structure [8].

Vitamin E Pathway in ROS Clearance

- Increased ROS in skeletal muscles due to oxidative stress causes hyperthyroidism.

- Vitamin E is used to treat the condition as an antioxidant, by donation of electrons to the free oxygen radicals to neutralize and stabilize them. This process stops the chain reactions of ROS

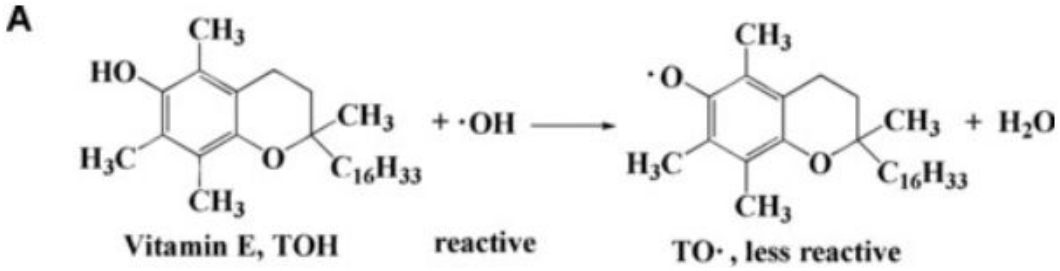


Fig.3: Direct reactions of vitamin E (TOH) with ▪OH [9].

Vitamin E and Hyperthyroidism

Oxidative stress is treated by vitamin E in different ways:

- **Regulation of gene expression;** encoding for glucose transporters (Slc2a1, Slc2a4) and factors involved in lipid metabolism and insulin signaling (Pparg, Ppara, Cd36) this way, vitamin E enhances glucose uptake and lipid metabolism, leading to improved insulin sensitivity (8).

- **Preservation of membrane integrity;** Vitamin E safeguards cell membranes by preventing lipid peroxidation, crucial for insulin signaling and glucose transportation, by preventing damage caused by free radicals(8)

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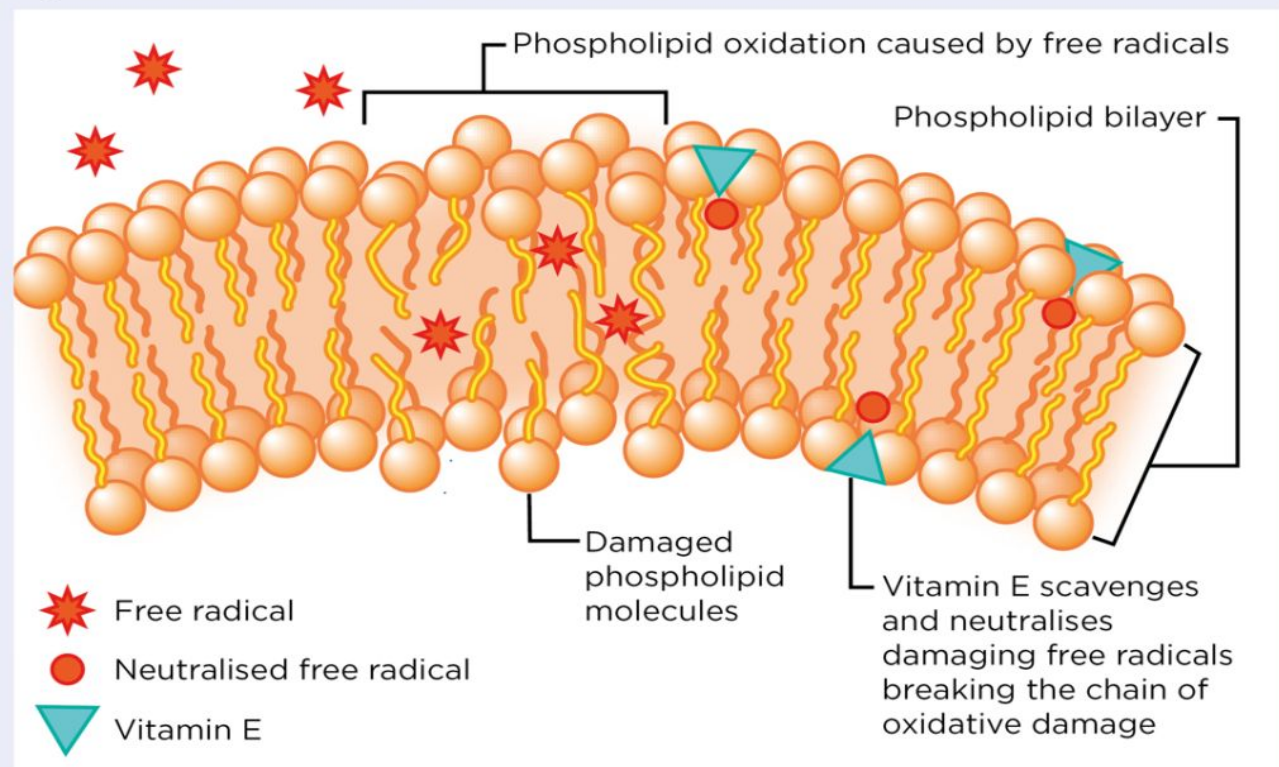


Fig.4: The role of vitamin E as an antioxidant on cell membrane [8].

Vitamin E chain-breaker antioxidant

- Alpha-tocopherol encounters a lipid peroxyl radical (LOO•) (highly reactive) and donates a hydrogen atom from its hydroxyl group to it which stabilizes the lipid peroxyl radical.

- Vitamin E itself becomes a tocopherol radical (•E), losing one of its hydrogen atoms. The cell has a system that regenerates Vitamin E back to its active antioxidant form by Another antioxidant,(Vitamin C)(ascorbic acid), donates an electron to the tocopherol radical (•E) restoring Vitamin E antioxidant activity. (10)

- This regeneration process ensures that Vitamin E can continue its role as a chain-breaking antioxidant. (10)

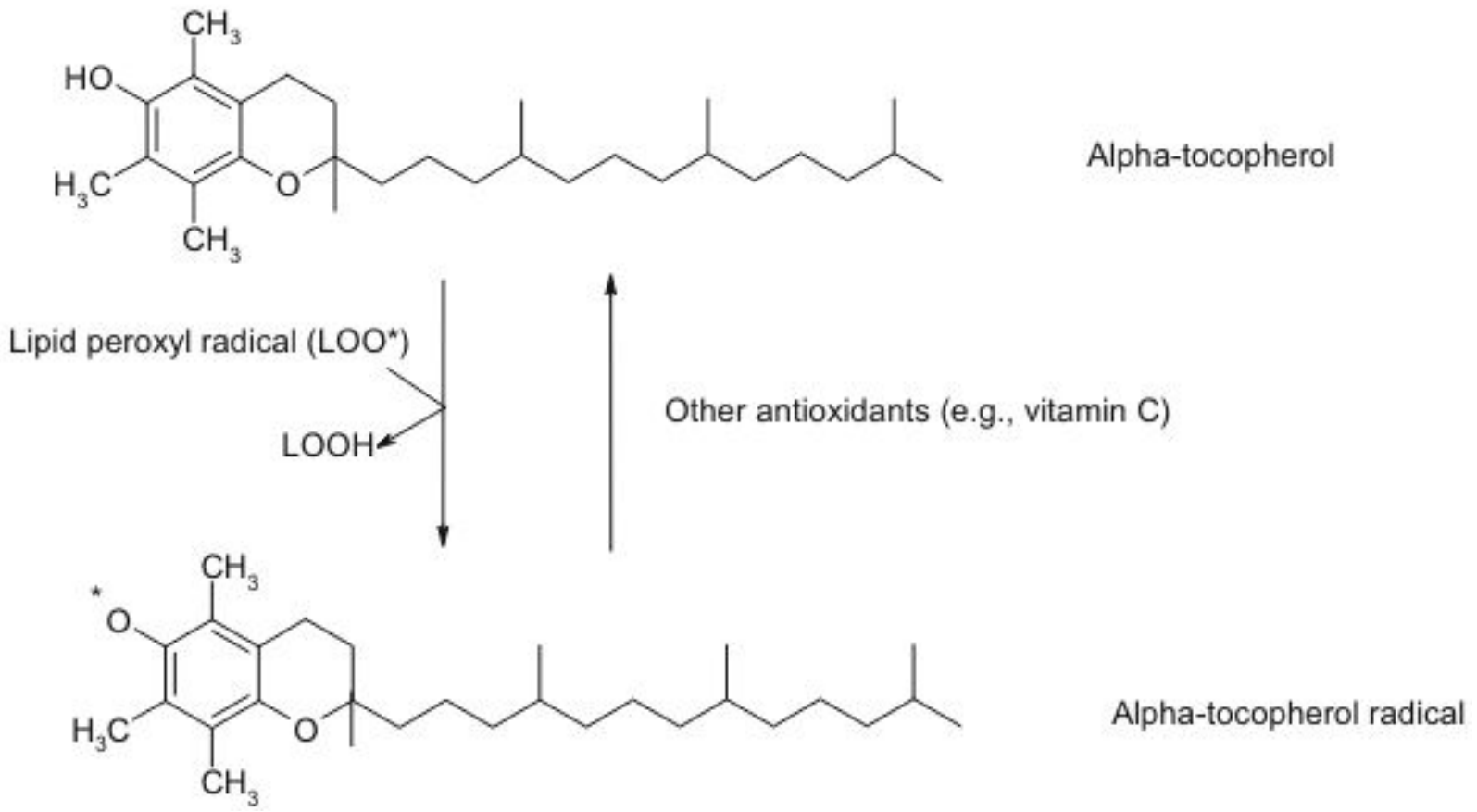


Fig.5: Antioxidant role of alpha tocopherol in scavenging radicals in lipid peroxidation [10].

Cellular Stress Responses

This pathway examines cellular stress responses, focusing on key proteins like EIF2α, JNK, PGC-1α, BIP, and NRF1. It investigates EIF2α phosphorylation, regulatory mechanisms of PGC-1α, and roles of BIP in ER protein folding and NRF1 in mitochondrial stress adaptation. The goal is to understand cellular adaptation to stress and identify therapeutic targets for stress-related diseases [11,12].

Impact of eIF2α Phosphorylation on Protein Synthesis and Cellular Stress Response

- **Phosphorylation of eIF2α at Serine-51:** Phosphorylation of eIF2α at Serine-51 is a key regulatory event in stress response pathways. It inhibits eIF2B, reducing the formation of the active eIF2-GTP complex, thereby attenuating global protein synthesis [11]
- **Formation of Stress Granules:** conserves energy and nutrients during stress by triggering the formation of stress granules. These specialized cytosolic foci manage mRNA regulation and mRNA-protein interactions [13].
- **Impact on NF-κB Activation:** The accumulation of P-eIF2α leads to reduced synthesis and increased turnover of IκBα. This results in the activation of NF-κB, its nuclear translocation, and targeted transcriptional regulation.

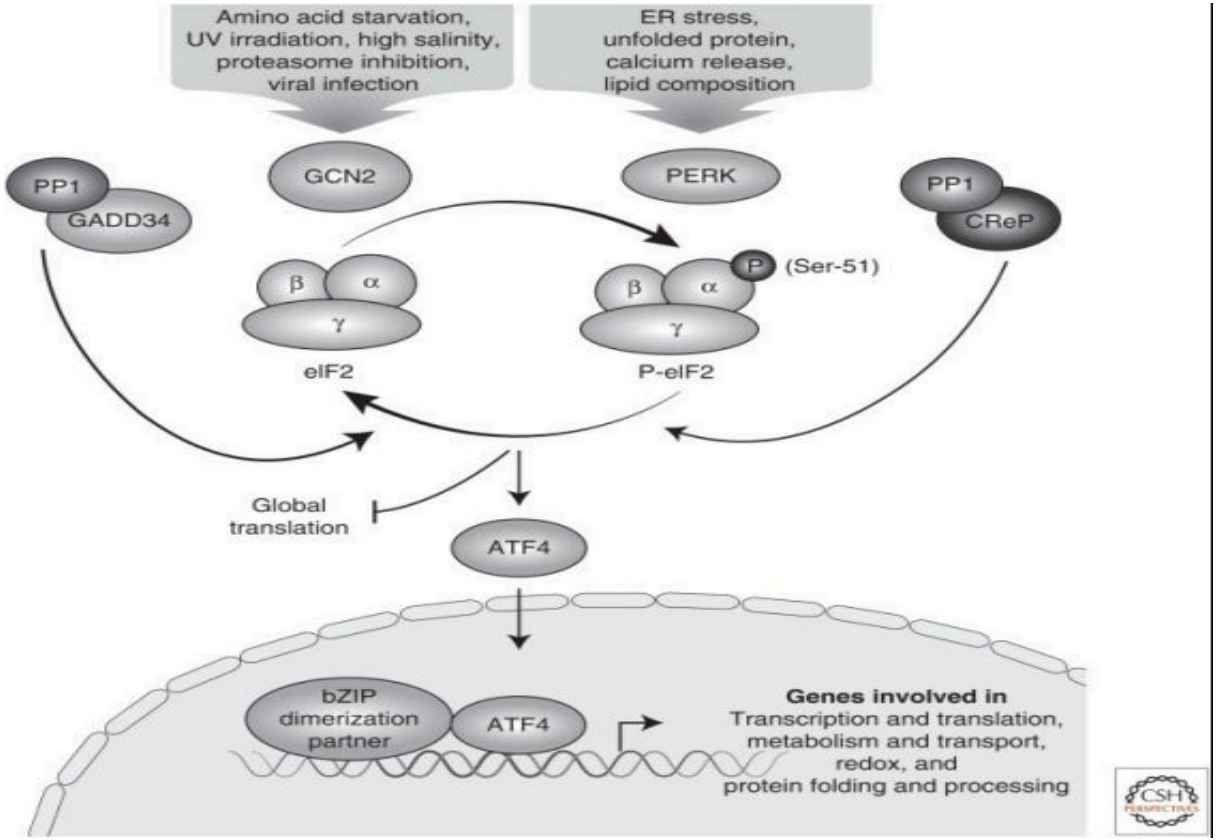


Fig.6: Phosphorylation of eIF2α by kinases GCN2 and PERK regulates translation. PP1c/CReP dephosphorylate eIF2α in basal conditions, while GADD34 mediates feedback control. Phosphorylation reduces global translation, promoting ATF4 translation. ATF4 regulates ISR gene transcription for stress adaptation[12]

Redox Regulation of Protein SUMOylation & Ubiquitination:

- Investigated biochemical interactions between oxidative stress and mitochondrial biogenesis[14,15]
- Analyzed alterations in PGC1-α, NRF1, and BIP levels, along with EIF and JNK phosphorylation dynamics[15]
- Findings revealed complex relationships between oxidative stress and changes induced by thyroid hormone treatment.

Vitamin E Supplementation Effect: In thyroid hormone-treated rats supplemented with vitamin E (H + VE), reductions in NRF1 and BIP content were noted.Additionally, vitamin E supplementation led to diminished phosphorylation of EIF and JNK.[14]

Oxidative Stress and Regulation: Oxidative stress influences key regulators of mitochondrial biogenesis, namely PGC1-α, NRF1, and BIP.The phosphorylation status of EIF and JNK, pivotal for protein translation and stress response, is altered by oxidative stress.[15]

Crucial Interplay for Cellular Homeostasis: The interplay between these factors is crucial for maintaining cellular homeostasis and effective response to stressors.

RESEARCH GAPS

- Research on insulin resistance in hyperthyroid animals' livers is limited, with conflicting data on the link between oxidative stress and insulin resistance in skeletal muscle.

- Further investigation is needed to understand the mechanisms behind hyperthyroidism-induced oxidative stress and vitamin E supplementation's impact on insulin sensitivity.

- Vitamin E supplementation may increase insulin sensitivity, but its effect on oxidative damage and gene expression in skeletal muscle is still unknown.

Methodology

Research paradigm	Procedures
Data Analysis and Expected Results	Budget

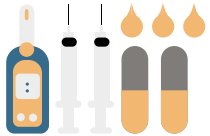
Research paradigm

The experimental study will follow a quantitative paradigm as multiple tests will be done and statistically analyzed. Additionally, the study involves comparing various variables and factors between the control and experimental groups to determine relationships and patterns within the data.

Procedures

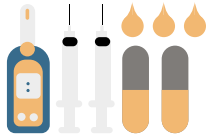
Animals

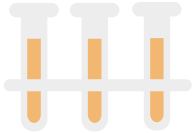
Isolation of Muscle Mitochondria



Glucose Tolerance Test

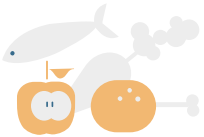
Oxidative Damage Assessment and In Vitro Susceptibility to Oxidative Stress (16,17,18)





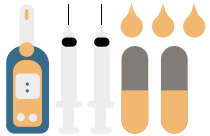
Tissue Preparations

Total ROS Content (19)

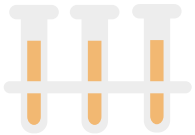


Procedures

NADPH Oxidase (NOX) Activity Assay (20,21)



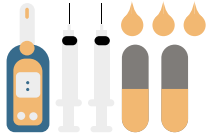
H2O2 Mitochondria l Release (22)



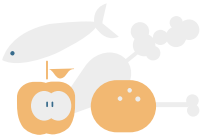
Antioxidant Enzymes Activities (23,24,25)

Tissue and Mitochondrial Respiration

Response of Skeletal Muscle to Insulin and Immunoblotting Analyses (26)



RNA Isolation, RT-PCR, and qPCR



Data Analysis and Expected Results

Data analysis process:

- Presenting biochemical and blotting analyses as means ± SEM
- Conducting experiments in technical duplicate or triplicate
- Using Tukey's pairwise comparison tests and one-way ANOVA
- Classifying differences with p values ≤ 0.05 as significant (27)
- Analyzing data with GraphPad Prism 8.0.2
- Determining normal data distribution in qPCR reactions using the Shapiro-Wilk test
- Assessing statistical significance between control and testing samples or different groups using a two-tailed Student's t-test

Data Analysis and Expected Results

Expected outcomes of Vitamin E supplementation in hyperthyroid rats:

- Potential improvement in insulin sensitivity
- Protection against oxidative damage in skeletal muscle and modulation of antioxidant enzyme activity
- Influence on mitochondrial function and energy production
- Potential link between cellular stress response, insulin resistance, and thyroid hormone treatment, with Vitamin E potentially mitigating these effects
- Enhancement of expression of muscle glucose transporters and regulation of inflammation in skeletal muscle
- Modulation of genes involved in muscle lipid balance and insulin signaling (28,29,30)

Budget

Name	Price	Name	Price
Research lab Rent	Total rental costs:$100 (glucometer) + $150 (glucose tolerance test equipment) + $200 (balance) + $300 (centrifuge) + $400 (fluorometer) + $500 (multimode microplate reader) + $100 (Potter Elvejem homogenizer) = $1,650 per month= 82500 EGP	**Costs for salaries and other general expenses associated with the research**	The salary costs could range from 250$ =12500 EGP to 500$ =25000 EGP per person, depending on the hourly or daily rates
Wistar rats 50-74g weight, 21-25 approx days	15 $ each so 15$*32(number of rats)=960$= 48000 EGP for all	**Standard diet (2 Lb. Bag)** (31)	19.6$=982EGP
α-tocopherol (Vitamin E) 10 mg	60$=3000EGP	**Zoletil (for euthonasia) 50/50 mg/ml**(32)	39.90$=1995EGP

Budget

The total budget is 7545.5 $ (377275 EGP) including 5% extra allowance.

Homogenization solution **20 mL(33)**	70.00$=3500 EGP	**insulin 10 mL**	1.3$=63.5 EGP
Commercial ELISA kit (uncoated) **(2 kits will be used)**	300$ each so Approximately 2*300$=600$ =30000 EGP for all	**Krebs solution** **1 L**	160.00$=8000 EGP
"TRIzol Reagent **100ml** (34)	86$=4300 EGP	**qPCR MasterMix** **Includes: This master mix comes in a 250µL tube, enough for 25 reactions.**	77$=3850 EGP
Publication fees	2000$=100000 EGP	**GraphPad Prism 8.0.2** (35)	Free trial for 1 month on official website

Table [1]: Budget of the proposed study

References

1. World. Diabetes [Internet]. Who.int. World Health Organization: WHO; 2023 [cited 2024 May 11]. Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes
2. Hong Y, Boiti A, Vallone D, Foulkes NS. Reactive Oxygen Species Signaling and Oxidative Stress: Transcriptional Regulation and Evolution. Antioxidants [Internet]. 2024 Mar 1 [cited 2024 May 12];13(3):312–2. Available from: https://www.mdpi.com/2076-3921/13/3/312
3. Zhang L, Wang X, Cueto R, Comfort Effi, Zhang Y, Tan H, et al. Biochemical basis and metabolic interplay of redox regulation. Redox biology [Internet]. 2019 Sep 1 [cited 2024 May 12];26:101284–4. Available from: https://pubmed.ncbi.nlm.nih.gov/31400697/
4. Klaudia Jomova, Raptova R, Alomar SY, Alwasel SH, Nepovimova E, Kuca K, et al. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Archives of toxicology [Internet]. 2023 Aug 19 [cited 2024 May 12];97(10):2499–574. Available from: https://link.springer.com/article/10.1007/s00204-023-03562-9
5. Zhang J, Wang X, Vikash Vikash, Ye Q, Wu D, Liu Y, et al. ROS and ROS-Mediated Cellular Signaling. Oxidative medicine and cellular longevity [Internet]. 2016 Jan 1 [cited 2024 May 12];2016:1–18. Available from: https://pubmed.ncbi.nlm.nih.gov/26998193/

6. Voldstedlund M, Tranum-Jensen J, Handberg A, Vinten J. Quantity of Na/K-ATPase and glucose transporters in the plasma membrane of rat adipocytes is reduced by in vivo triiodothyronine. Eur J Endocrinol. 1995;133: 626-634.
7. Goto H, Sumida Y, Nakatani K, Yano Y, Shima T: Effect of triiodothyronine on glucose transport in rat adipocytes. Life Sciences, 61: 193-204, 1997.
8. Lü JM, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. Journal of Cellular and Molecular Medicine [Internet]. 2009 Sep 14;14(4):840–60. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2927345/
9. Bayram-Weston JK Maria Andrade-Sienz, Zubeyde. Vitamins E and K: their role and the effects of deficiency [Internet]. Nursing Times. 2024 [cited 2024 May 19]. Available from: https://www.nursingtimes.net/clinical-archive/nutrition-and-hydration/vitamins-e-and-k-their-role-and-the-effects-of-deficiency-22-04-2024/
10. Nakamura YK, Omaye ST. Vitamin E-modulated gene expression associated with ROS generation. Journal of Functional Foods. 2009 Jul;1(3):241–52.
11. Back SH. Roles of the Translation Initiation Factor eIF2α Phosphorylation in Cell Structure and Function. Cell Structure and Function. 2020;45(1):65–76
12. Wek RC. Role of eIF2α Kinases in Translational Control and Adaptation to Cellular Stress.Cold Spring Harbor Perspectives in Biology. 2018 Feb 12;10(7):a032870.
13. Hock MB, Kralli A. Transcriptional Control of Mitochondrial Biogenesis and Function.Annual Review of Physiology. 2009 Mar;71(1):177–203

14. Weitzel JM, K. Alexander Iwen. Coordination of mitochondrial biogenesis by thyroid hormone. Molecular and cellular endocrinology [Internet]. 2011 Aug 1 [cited 2024 May 12];342(1-2):1–7. Available from: https://pubmed.ncbi.nlm.nih.gov/21664416/
15. Venditti P, Bari A, Di Stefano L, Cardone A, Della Ragione F, D'Esposito M, et al.Involvement of PGC-1, NRF-1, and NRF-2 in metabolic response by rat liver to hormonal and environmental signals. Molecular and Cellular Endocrinology [Internet]. 2009 Jun 16[cited 2024May12];305(1-2):22–9.Available From:https://pubmed.ncbi.nlm.nih.gov/19433258/
16. Heath RL, Tappel AL. A new sensitive assay for the measurement of hydroperoxides. Analytical biochemistry. 1976 Nov 1;76(1):184-91.
17. Mesquita CS, Oliveira R, Bento F, Geraldo D, Rodrigues JV, Marcos JC. Simplified 2, 4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Analytical biochemistry. 2014 Aug 1;458:69-71.
18. Fasciolo G, Napolitano G, Aprile M, Cataldi S, Costa V, Ciccodicola A, Di Meo S, Venditti P. Hepatic insulin resistance in hyperthyroid rat liver: Vitamin E supplementation highlights a possible role of ROS. Antioxidants. 2022 Jun 29;11(7):1295.
19. Driver AS, Kodavanti PR, Mundy WR. Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicology and teratology. 2000 Mar 1;22(2):175-81.
20. Suzuki Y, Lehrer RI. NAD (P) H oxidase activity in human neutrophils stimulated by phorbol myristate acetate. The Journal of Clinical Investigation. 1980 Dec 1;66(6):1409-18.
21. Minkenberg I, Ferber E. Lucigenin-dependent chemiluminescence as a new assay for NAD (P) H-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. Journal of immunological methods. 1984 Jun 8;71(1):61-7.

22. Hyslop PA, Sklar LA. A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. Analytical biochemistry. 1984 Aug 15;141(1):280-6.
23. Flohé L, Günzler WA. [12] Assays of glutathione peroxidase. InMethods in enzymology 1984 Jan 1 (Vol. 105, pp. 114-120). Academic Press.
24. Carlberg IN, Mannervik BE. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. Journal of biological chemistry. 1975 Jul 25;250(14):5475-80.
25. Aebi, H. Catalase in vitro. Methods Enzymol. 1984, 105, 121–126.
26. Amouzou C, Breuker C, Fabre O, Bourret A, Lambert K, Birot O, Fedou C, Dupuy AM, Cristol JP, Sutra T, Molinari N. Skeletal muscle insulin resistance and absence of inflammation characterize insulin-resistant grade I obese women. PLoS One. 2016 Apr 25;11(4):e0154119.
27. Gianluca Fasciolo, Napolitano G, Aprile M, Cataldi S, Costa V, Teresa M, et al. Muscle Oxidative Stress Plays a Role in Hyperthyroidism-Linked Insulin Resistance. Antioxidants [Internet]. 2023 Feb 27;12(3):592–2.
28. Napolitano, G.; Fasciolo, G.; Di Meo, S.; Venditti, P. Vitamin E Supplementation and Mitochondria in Experimental and Functional Hyperthyroidism: A Mini-Review. Nutrients 2019, 11, 2900. [CrossRef]
29. Lin, H.Y.; Weng, S.W.; Chang, Y.H.; Su, J.; Chang, M.; Tsai, C.J.; Shen, F.C.; Chuang, J.H.; Lin, T.K.; Liou, W.; et al. The causal role of mitochondrial dynamics in regulating insulin resistance in diabetes: Link through mitochondrial reactive oxygen species. Oxid. Med. Cell Longev. 2018, 2018, 7514383. [CrossRef]

30. Lark, D.S.; Fisher-Wellman, K.H.; Neufer, P.D. High-fat load: Mechanism(s) of insulin resistance in skeletal muscle. Int. J. Obes. 2012, 2 (Suppl. S2), S31–S36. [CrossRef] [PubMed].
31. Mazuri Vegetarian Rat & Mouse Diet - 2 Pound Bag Egypt | Ubuy [Internet]. Ubuy Egypt.[cited 2024 May 13].Available From: https://www.ubuy.com.eg/en/product/2NH6VSPE-mazuri-rat-amp-mouse-diet-rodent-food-2-pound-bag
32. Zoletil 50- Injection for cats and dogs | Virbac [Internet]. PETBUCK. [cited 2024 May 13]. Available from: https://petbuck.net/products/zoletil-50-injection-for-cats-and-dogs-virbac
33. QuantiGeneTM Homogenizing Solution [Internet]. www.thermofisher.com. [cited 2024 May 13]. Available from: https://www.thermofisher.com/order/catalog/product/QS0518
34. TRIzolTM Reagent [Internet]. www.thermofisher.com. Available from: https://www.thermofisher.com/order/catalog/product/15596026
35. Prism 8.0.2 Release Notes [Internet]. Graphpad.com. 2019 [cited 2024 May 13]. Available from: https://www.graphpad.com/updates/prism-802-release-notes

Thank You

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