

Comparative effect of vitamin E compounds on HSP70 expression in response to acute redox imbalance in Chang liver cells

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Research

Keywords: HSP70, antioxidant, vitamin E, tocopherol, tocotrienol

Posted Date: April 21st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-22336/v1>

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Abstract

Background: HSP70 represents the most highly induced member of the stress protein family which is constitutively expressed in normal cell conditions as chaperon and rapidly upregulated in response to stress conditions. Vitamin E compounds are considered to be the most potent oxidation chain breaking compounds. Although α -tocopherol, has been long considered as the most potent antioxidant among the vitamin E isomers, many studies have recently suggested a higher antioxidant potency of tocotrienols compared to tocopherols. Present study was carried out to investigate the effects of tocotrienol supplementation compared to α -tocopherol on HSP70 expression.

Methods: To assess whether the increase in HSP70 secondary to the stress caused by the oxidant could decrease in the presence of vitamin E; two groups of cells were incubated for 4 hours with α -tocopherol and palm tocotrienol-rich fraction respectively with concentration of 10, 20 and 30 $\mu\text{g/ml}$ after the cells were pre-exposed to oxidative stress.

Results: The comparison between the negative control which represents the basal expression of HSP70 and the positive control that represents the expression of HSP70 as a response to the oxidant, has clearly indicated that treatment with 100 μM copper sulphates for 24 hours resulted in a significant up-regulation of HSP70 expression. The present study has shown that the overall effect of vitamin E compounds seems to reduce the increase in HSP70 expression. α -tocopherol tended to trigger a more constant rate of response to the dose given but unable to significantly induced the decrease of the HSP70 expression as compared to tocotrienol.

Conclusion: Treatment with vitamin E has conferred significant protection against stress induced cellular damage. Whether the reduction of HSP70 by vitamin E is deleterious or beneficial is still controversial and needs to be considered.

Introduction

Cells are adapting to changes in environment continually which resulting in regulation of gene expression to readjust to new physiological situations. Some of these changes may cause cellular integrity and prompt the stress response. Expression of heat shock proteins (HSP) is a general response to a variety of chemical, physical and physiological stresses, which can be directed at conserving cell integrity for survival. HSP belong to a family of well-conserved proteins and classified according to molecular weights: Hsp60, 70, 90, 100, and small HSP (1). Some of HSPs participate in several basic processes and present within the cells constitutively, such as stabilization of substrates, protein folding and assembly of macromolecule structures (2). Upon stress conditions, expression of HSP is induced to participate in folding of degraded and denatured polypeptides as a consequence of insult. Furthermore, HSP can stabilize numerous cellular processes affected by stress conditions including transcription, splicing, translation and transport. Also, damaged macromolecules which are under repair and cellular debris which need to be disposed, requiring HSP for their process (3).

Oxidative stress induces adaption in expression of protective enzyme and heat shock proteins (HSPs) in a variety of tissues. HSP70 represents the most highly induced member of the stress protein family and it is suggested to have significant cytoprotective effects against a variety of stressors. Vitamin E is the major soluble, chain breaking antioxidant in the body, comprises eight structurally related lipid soluble compounds, α -, β -, γ - and δ -tocopherol; and α -, β -, γ - and δ -tocotrienol. Little is known about the effect of vitamin E compounds on expression of HSP70 in oxidative stressed cells. The aim of this study is to examine the influence of oxidative stress on HSP70 expression and to compare the effects of α -tocopherol and α -tocotrienol on oxidative-influenced HSP70 in Chang liver cells.

This study is a contribution to the ongoing research on heat shock proteins, particularly HSP70, as the most remarkable member of the stress proteins. HSP70 offers great potential as a therapeutic target in diverse range of human disease pathologies associated with trauma, stress, and the expression of damaged and misfolded proteins. Therefore, better understanding of the conditions which lead to the reduction or increase of HSP70 expression will certainly assist in development of future therapeutic treatments.

Moreover, this study aids the growing concern on understanding the potential properties of tocotrienols compared to tocopherols. The abundance of α -tocopherol in human body and the comparable efficiency of all vitamin E molecules as antioxidants, led biologists to neglect the non-tocopherol vitamin E molecules as topics for basic and clinical research. Work on tocotrienols account for only 1% of the total literature on vitamin E (4). In conclusion, the effect of both TRF and α -tocopherol was examined and contrasted. The understanding of the influence of vitamin E compounds on cell HSP70 expression will provide invaluable tools to unravel further the potential of the cells for protection against stressful conditions.

Methodology

The cryopreserved human liver cell line CCL-13 (Chang liver, CHL; an immortalized non-tumor cell line derived from normal liver, purchased cryopreserved from the American Type Collection (A.T.C.C.)) were thawed and cultured in DMEM (Dulbecco's modified Eagle's medium) (GIBCO®), supplemented with 15% (v/v) foetal bovine serum and 50 U of penicillin - 50 μ g/ml of streptomycin solution (GIBCO®); in T-75 cell culture flask (NUNC®) and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Upon reaching 80-90% confluence, the cells were subcultured. Haemocytometer counting was performed to estimate the total cell number of cultured cells. Table 1 summarizes these findings.

Treatment of cells: The cells were induced for oxidative stress when they reached 70-80% confluence, by incubating the cells with 100 μ M CuSO₄ for 24 hours at 37°C and 5% CO₂. The medium was then removed and replaced with fresh medium containing either α -tocopherol or α -tocotrienol rich fraction with concentration of 10, 20, 30 μ g/ml for another 4 hours at 37°C. Cells incubated with 100 μ M CuSO₄ without antioxidants were used as positive control, whereas cells with no oxidant or antioxidant applied were

used as negative control. Ten T-75 cell culture flasks were used, two for the negative control, two for the positive control, three for natural α -tocopherol treatment, and three for α -tocotrienol fraction treatment.

Total cellular RNA was extracted based on the single-step method of RNA isolation by acid Guanidinium-Phenol-Chloroform extraction as described by Chomczynski & Sacchi (5). All glassware, plasticware including tubes and micropipette tips were soaked in DEPC treated water and autoclaved to reduce RNase contamination. Concentration of RNA was quantified by using Eppendorf Biophotometer and quality was analysed by gel electrophoresis. As RNase is prevalent in the environment, hence the conversion of RNA to cDNA should be done immediately following the quality control assessment to preserve RNA integrity.

In present study, the optimal concentration of $MgCl_2$ and DNA polymerase for the primer was at 1.5mM of $MgCl_2$, and 2.5 units of Taq DNA Polymerase respectively (the normal range of Taq DNA Polymerase used was between 1-1.5 units).

RT-PCR was performed to compare the expression of HSP70 mRNA in Chang liver cells in response to the different levels of Palm-Tocotrienol rich fraction and α -tocopherol in presence of exogenous oxidant. Gene-specific primer was designed by First BASE laboratories SDN BHD.

Statistical Analysis

To assess the significance of differences in expression of HSP70, the unpaired Student's t test was carried out by using the Minitab 18 software. Data were expressed as Mean \pm SEM. $p < 0.05$ were considered significant.

Result

Chang liver cells were incubated at 37°C with 100 μ M $CuSO_4$ for 24 hours. Then incubated at 37°C with palm TRF (10, 20, 30 μ g) or α -tocopherol (10, 20, 30 μ g). tcRNA was extracted by using the acid Guanidinium-Phenol-Choloroform extraction, and RT-PCR semi quantitative analysis was performed to examine HSP70 expressions. Gene expression was determined by measuring the integrated density values (IDV) of PCR product bands. In absence of the antioxidant, HSP70, expression increased significantly. Palm-TRF significantly reduced HSP70 expression at 20 μ g and 30 μ g and was more effective than tocopherol (10, 20, 30 μ g). Reduction of HSP70 expression is possibly attributed to the antioxidant properties of vitamin E compounds. Hence, this finding suggests that TRF is a more potent antioxidant than α -tocopherol in vitro. The present study adds to the growing evidence of the greater potency of palm-TRF as radical scavengers compared to tocopherols. Whether oxidative-induced HSP70 down-regulation is beneficial for the cell is still elusive. Further studies on the mechanism by which TRF and tocopherols suppress HSP70 expression and how this influence stressed cells may provide more conclusive explanation.

Discussion

Expression of Heat Shock Protein 70 in Oxidative Stress

In present research, the comparison between the negative control which represents the basal expression of HSP70 (31530597.5 ± 923268 ; $n=4$), and the positive control that represents the expression of HSP70 as a response to the oxidant (4094970.25 ± 10449032 ; $n=4$), has clearly indicated that treatment with 100 μM copper sulphates for 24 hours resulted in a significant up-regulation of HSP70 expression ($p= 0.04$), Figure 1 & Table 2). Overexpression of inducible HSP70 by oxidants has been reported by many other studies (6,7).

The mechanism by which this happens is still elusive, however it is suggested that HSP70 was induced secondary to the adverse effects of oxidative stress on protein conformation (8,9). The oxidative stress here is inducing as a result of increased copper sulphate level which is known to cause oxidative stress due to the overproduction of free radicals particularly hydroxyl radicals. These free radicals attack major cellular molecules and exhaust the antioxidant enzymes inside the cells (10). CuSO_4 can be reduced from Cu^{+2} to Cu^+ by apoplastic ascorbate and/or other electron donors. Cu^+ formed can participate in a Fenton reaction with apoplastic H_2O_2 to form the highly reactive hydroxyl radical ($^{\circ}\text{OH}$) (11). Consequently, being attack by ROS, proteins may be oxidized, causing carbonylation and aromatic hydroxylation of some amino acids (12). Furthermore, it has been indicated by some studies that oxidation of protein thiols may result in formation of non-native intermolecular disulphide. These alterations in protein structure collectively, can cause proteins to destabilize, denature and unfold so hydrophobic domains are exposed. This would be probably initiating a signal for induction of the heat shock response (13). This has been shown in earlier studies that demonstrated a rapid induction of cytosolic HSP70 expression following the injection of denatured proteins into the cells (14).

Transcription of human HSP70 in response to accumulated denatured and abnormally folded proteins is mediated through the activation of heat shock transcription factor (HSF1). Biochemical and genetic studies have clearly demonstrated critical roles for mammalian heat shock factor 1 (HSF1) in stress-inducible HSP gene expression (15). Whether HSF1 directly senses stress or is regulated by an upstream signalling cascade is not yet fully understood. However, what is clear is that in non-stressed conditions – when HSP70 levels are in excess- HSF1 is found in the cytoplasm in a non-DNA binding monomeric form, by virtue of its being complexes with HSP70. In the case of oxidative stress, HSP70 recognizes the abnormally folded proteins, possibly through the recognition of the hydrophobic regions, forming longer lived complexes. As a result, HSF1 is liberated from HSP70; phosphorylated and converted to a trimer with the capacity to bind DNA. HSF then translocates from cytoplasm to nucleus where it binds to heat shock elements (HSE) in the promoter region of HSP70 coding genes, and subsequently activates HSPs transcription (16).

Consequently, it can be suggested that the significant induction of heat shock protein 70 mRNA observed in the present study, is possibly attributed to the accumulation of damaged proteins as a result of oxidative stress, and this was mediated, at the transcriptional level, by HSF1.

The induction of HSP70 following oxidative stress appears to be a part of cellular protective mechanisms to protect against subsequent stress, apparently by preventing or repairing stress-induced protein denaturation and promoting protein folding thus enhancing cells capability to survive (17,18). HSP70 was also shown to effectively inhibit the cellular death processes (19,20). The HSP70 seems to increase resistance against oxidative stress-mediated apoptosis. Reports revealed that HSP70 inhibits apoptosis induced by ethanol, hydrogen peroxide, TNF, UV radiation and several chemotherapeutic agents. All of these treatments have been found to produce ROS within cells during their apoptotic induction (21–23).

The mechanisms by which HSP70 confer protection from ROS and free radicals are not completely understood, but recent studies suggest several possible mechanisms such as : (1) inhibiting protein misfolding and aggregation (24); (2) refolding abnormally folded proteins (25); (3) directing damaged proteins to lysosomes (26), or to peroxisomes to be degraded (27); (4) increasing the expression or the activity of endogenous scavengers of ROS (28); (5) and it also seems to protect DNA, mitochondria from free radicals (29,30).

HSP70 Expression Level in Presence of Vitamin E

To assess whether the increase in HSP70 secondary to the stress caused by the oxidant could decrease in the presence of vitamin E; two groups of cells were incubated for 4 hours with α -tocopherol and palm tocotrienol-rich fraction respectively with concentration of 10, 20 and 30 $\mu\text{g/ml}$ after the cells were pre-exposed to oxidative stress.

The present study has shown that the overall effect of vitamin E compounds seems to reduce the increase in HSP70 expression (Figure 1). α -Tocopherol tended to trigger a more constant rate of response to the dose given but unable to significantly induced the decrease of the HSP70 expression as compared to TRF (see Figures 1 and Table 3). This observation was supported by some other studies. Topbas and colleagues (31) demonstrated that in liver cells, HSP70 that was induced as a result of vitamin E deficiency, was significantly reduced following re-supplementation with vitamin E. Similarly, vitamin E, particularly α -tocopherol, has been shown to reduce HSP70 levels in human muscles and blood following exercise respectively (32,33), in leukocytes after treadmill running and in human skin fibroblasts after oxidative stress (35).

The mechanism involved in this reduction is not well understood. However, this effect is possibly attributed to the potent antioxidant properties of vitamin E (32). It is likely that the effect of vitamin E on HSP70 was mediated through the scavenging of free radicals and ROS generated by the oxidant and its ability to break radical-propagated chain reactions. As HSF1 is sensitive to redox, it would be imperative to suggest that scavenging of free radicals and the subsequent reduction of the oxidative stress state of cells had possibly inhibited the activation of this factor, and consequently HSP70 (32,33).

Since the 1960s, vitamin E potent antioxidant function had been clearly recognized (36). The strong reducing capacity of vitamin E is related to its chemical structure. All vitamin E isomers have active hydroxyl groups attached to benzene ring-based structure and a hydrophobic side chain. It is the hydroxyl

group that involves in reducing the free radicals since it can easily donate a hydrogen atom, leading to the formation of vitamin E radicals (chromanoxyl) (37). These chromanoxyl radicals are relatively stable and can be reduced back to vitamin E form by compounds naturally present in biological systems such as ascorbic acid and lipoic acid, in a process termed “recycling” of vitamin E (38). On the other hand, vitamin E side chains are important for the mobility and the incorporation of the vitamin molecules within the membrane phospholipids bilayer (39,40).

As a result of, the present study shows that vitamin E had maintained the reducing environment of the cell, hence prevented the activation of HSF1 and in turn HSP70 by ROS and other free radicals.

Comparative Effect of α -Tocopherol and Palm-Tocotrienol Rich Fraction on HSP70 Expression

With reference to the positive control (Table 3) HSP70 mRNA was significantly reduced by palm-TRF at 20 μ g and 30 μ g ($p=0.028$, 0.032 respectively; $n=4$), and this effect was more pronounced at 20 μ g palm-TRF (Table 3). In comparison with the negative control (Table 3), at these concentrations of palm-TRF, HSP70 expression was down-regulated to its basal levels ($p=0.051$, 0.224 respectively; $n=4$).

Palm-TRF also appeared to be more effective in reducing HSP70 expression than α -tocopherol (10, 20, 30 μ g). Although α -tocopherol (10, 20, 30 μ g) had caused a decline in HSP70 expression in a dose dependent manner (Figure 1), this decrease was not statistically significant compared to the positive control ($p=0.814$, 0.224 , 0.052 respectively, see Table 3). However, at 30 μ g α -tocopherol, HSP70 expression was almost similar to the negative control ($p=0.326$).

The tocotrienol-rich-fraction (TRF) of palm oil has been anticipated as an efficient alternative for α -tocopherol due to its potent antioxidant properties in vitro (41–45). Although there was no report on TRF effect on HSP70, the present study findings are in agreement with a number of studies that suggest a higher antioxidant potency of tocotrienols compared to tocopherols in vitro; as HSP70 reduction has been earlier related in this discussion to vitamin E antioxidant function. It has been reported that α -tocotrienol exhibited significantly greater peroxy radical scavenging potency than α -tocopherol in phosphatidylcholine liposomes (46). Furthermore, palm-TRF has been also shown to be a more effective inhibitor of LDL oxidation in endothelial cell lipid peroxidation than α -tocopherol in vitro (47). However, in contrast to these findings, a small number of studies suggested that tocopherols and tocotrienols exerted the same reactivity toward radicals and the same antioxidant activities against lipid peroxidation in solution and liposomal membranes. These conflicting findings may be due to the differences in the systems used to conduct these studies (47).

Several reasons have been suggested for the increased antioxidant activity of tocotrienols compared to tocopherols, mostly related to the difference in the hydrophobic side chain saturation level. The unsaturated side chain of tocotrienols has been proposed to allow for more efficient penetration into tissues and increase in the rate of transfer of tocotrienols between liposomal membranes than tocopherols (48). It has been suggested that the higher antioxidant potency of tocotrienols is due to a higher recycling efficiency of the chromanoxyl radicals of the tocotrienols (tocotrienoxyl radical) in

membranes and lipoproteins, a more uniform distribution of tocotrienols in the cellular membranes, and stronger disordering effect on membranes than α -tocopherol. In addition, studies have indicated that α -tocotrienols located closer to the membrane surface, which may facilitate recycling (46). These properties are likely result in a more efficient interaction of the chromanol ring of tocotrienols with reactive oxygen species (46).

Despite the fact that the antioxidant efficiency of tocotrienols in vitro is higher than that of tocopherols, these compounds have shown to be less absorbed in vivo than α -tocopherol that is found to be the dominant vitamin E isomer in human plasma and tissue (49). Possible explanation for the reduced bioavailability includes (1) the presence of a transfer protein in the liver that specifically enriches VLDL with α -tocopherol leading to the secretion of these compounds from the liver in a manner that discriminates between tocopherols and tocotrienols (49), (2) less tissue retention and half-life (3) and the higher rate of tocotrienols metabolism compared to tocopherols (50).

Nevertheless, other studies have indicated that oral intake of tocotrienol makes it bioavailable to all vital organs; even though in significantly lower concentrations than α -tocopherol (51). Nanomolar tocotrienols has been demonstrated to be sufficient to exert antioxidant-independent protective effects in brain (48). The same study has suggested that at micromolar but not nanomolar α -tocotrienol can protect against chemically produced peroxy radicals (48,52). This effect has been clearly shown in this study.

Conclusion

Implication of Reduced HSP70 mRNA Levels on Stressed Cells

Whether the reduction of HSP70 by vitamin E is deleterious or beneficial to the stressed cell is still controversial and needs to be considered. The heat shock proteins vital role in cytoprotection against a variety of stressors that damage the cell and their role in cellular adaption to stress; may suggest that reduced expression by vitamin E may have abolished the adaptive response of stressed cells, or slightly reduced the protective effect of HSP70 (32). However, the findings of this study suggest that the observed effect was apparently secondary to the diminution of cell oxidative stress state due to the scavenging of free radicals and antioxidant properties of vitamin E, since so far there is no recognized mechanism shows the direct interaction between HSF1 or HSP70 and vitamin E. Also, there is a more reduced redox status when vitamin E is supplemented which possibly creates a condition within the cell that does not require additional HSP70 for its cellular protein repair caused by uncontrolled oxidative products.

It is also important to suggest that the presence of antioxidant nutrients in the stressed cell might help to accelerate the process of damaged or unfolded proteins repair accomplished by HSP70 (19). Consequently, less HSP70 will be needed. This supposition has been supported by some studies which have demonstrated that treatment with vitamin E has conferred significant protection against stress-induced modification of cellular sulfhydryl and carbonyl content. It is also suggested that although HSP70 was reduced, functional levels of cytoprotective HSP70 was maintained (35). Therefore, it is recommended to assess the protective effects of vitamins E in any future study by examining cell

viability and integrity in conditions when HSP70 is overexpressed and subsequent to the incubation with vitamin E.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financially supported by Universiti Putra Malaysia, Research University Grant Scheme (grant number: 04-02-12-1795RU).

Authors' contributions

SAHK¹, MSAM¹ and **HK²** were responsible for the study concept and design. **SAHK¹, MSAM¹** and **HK²** contributed to data acquisition and analysis. **SAHK¹** drafted the manuscript. **MSAM¹** and **HK²** provided critical revision of the manuscript for important intellectual content and approved final version for publication. All authors read and approved the final manuscript.

Acknowledgement

Not applicable

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Tables

Table 1: Chang liver cell count using haemocytometer

Test No.	Total cells count in all squares	Average cell count	Density cells/ml	Total cell Number (in 4 ml)
1	351	87.75	1.76 × 10 ⁸	7.02 × 10 ⁸
2	547	136.75	2.74 × 10 ⁸	10.94 × 10 ⁸
			Average	8.98 × 10⁸

Dilution Factor= 2; Number of squares = 4

Table 2: quantitation of the extracted tcRNA from Chang liver cells by the single step extraction method

Sampel	Absorbance (A260)	Absorbance (A280)	A260: A280	Concentration (µg/ml)
C1c	0.578	0.381	1.52	5785
C2d	0.115	0.068	1.69	1145
L1	0.314	0.242	1.34	3500
L3	0.373	0.217	1.71	3750
T10c	0.088	0.059	1.50	877
T20a	0.207	0.183	1.13	2069
T30d	0.956	0.870	1.10	9561
α10a	1.77	1.63	1.53	17758
α20a	0.06	0.037	1.63	597
α30d	0.524	0.432	1.21	5234

C1, C2 = positive control samples; L1, L3 = negative control samples; T10, T20, T30= samples treated with 10, 20, 30 µg TRF respectively; α10, α20, α30= samples treated with 10, 20, 30 µg tocopherol respectively; small letters a, b, c, d= samples number 1, 2, 3, 4 of each category.

Table 3: level of significance of difference between control samples and treatment samples

Sample	Level of Significance of Difference (p value)	
	CTRL+ (n=4)	CTRL- (n=4)
TRF 10 (n=4)	0.591	0.015*
TRF 20 (n=4)	0.028*	0.051
TRF 30 (n=4)	0.032*	0.224
α-Toc 10 (n=4)	0.814	0.006*
α-Toc 20 (n=4)	0.224	0.046*
α-Toc 30 (n=4)	0.052	0.326

CTRL+ = positive control samples; CTRL- = negative control samples; TRF10, TRF20, TRF30 = samples treated with 10, 20, 30 µg TRF respectively; α- Toc 10, ; α- Toc 20, ; α- Toc 30 = samples treated with 10, 20, 30 µg tocopherol respectively.

Values followed by * superscript were significantly different * p < 0.05

Figures

HSP70 Expression Mean±SEM

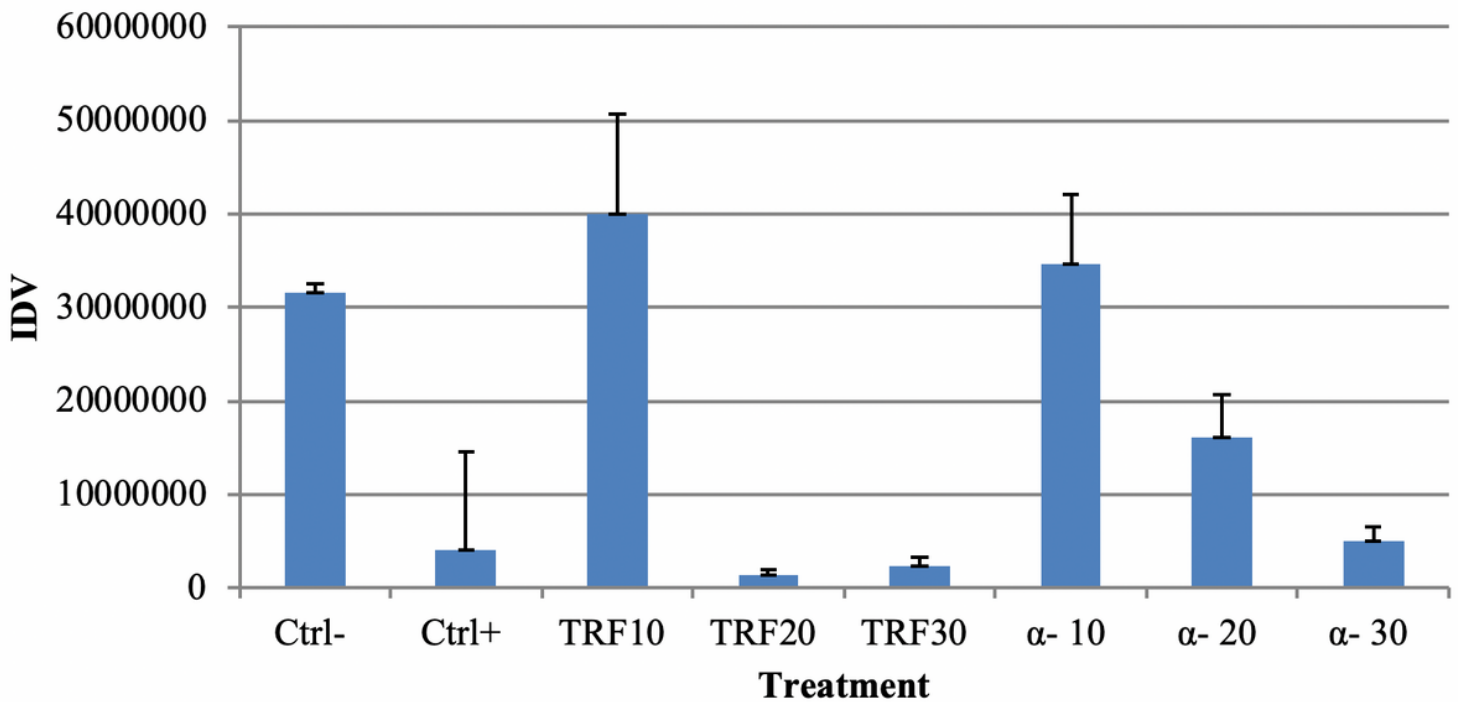


Figure 1

A graphical representation of the difference in the expression of HSP70. Ctrl+ : positive control samples
Ctrl- : negative control samples TRF 10, 20, 30: samples treated with 10, 20, 30 µg TRF respectively α-Toc
10, 20, 30: samples treated with 10, 20, 30 µg Toc respectively Values expressed as Mean ±SEM of the
IDV of each sample ($p < 0.05$).