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Vitamin D_3 and Alpha-Lipoic Acid alleviate chronic immobilization stress induced metabolic changes in adult male rats

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- Vitamin-D_3
- Alpha-lipoic-acid
- Chronic immobilization stress
- Metabolic derangement
- Sex hormones
- Antioxidant
- Antiapoptotic

Abstract

Context: Stress disturbs metabolic processes. Vitamin-D_3 and alpha-lipoic-acid (ALA) are known by their bone mineralizing and antioxidant effect respectively, with some metabolic effects. **Objective:** Elucidate the effect of Vitamin-D_3 and ALA on metabolic responses to chronic stress and clarify their underlying mechanism(s). **Materials and Methods:** 40 adult local strain albino male rats were randomly allocated into Control, Stressed, Vitamin-D_3- and ALA- treated stressed groups. Rats were subjected to determination of anthropometric, glycemic parameters, lipid profile, Malondialdehyde (MDA), total antioxidant capacity (TAC), nitrites, insulin, leptin, testosterone, estrogen, immunohistochemistry for caspase-3 of pancreatic tissue. **Results:** Upon vitamin-D_3 and ALA treatment, fasting plasma glucose and glucose output by kidneys, lipid profile, estrogen and caspase-3 area% were reduced, while glucose uptake by diaphragm, HOMA-B and testosterone were elevated when comparted with stressed group. Only ALA showed significantly elevated TAC and insulin when compared with stressed rats. Insignificant changes were shown between the treated groups, except for reduced testosterone and elevated estrogen in ALA-treated. **Conclusion:** Both treatments are effective in amelioration of glycemic and lipidemic derangements induced by chronic stress by restoration of normal sex hormones pattern, antioxidant and antiapoptotic effects, with more prominent antioxidant effect by ALA.

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INTRODUCTION

Stress, a state of altered homeostasis, is induced by either external or internal stimuli[(1)]. Repeated stress exposure often leads to disturbances in the internal environment, thereby increasing the risk of various health problems[(2)]. Stress was found to elevate blood glucose level and decrease insulin action[(3)], and to alter the lipid profile[(4)]. Although, there is some suggestion from retrospective human studies that stress can precipitate type I diabetes, animal studies are contradictory with different stressors either having excitatory or inhibitory effects upon the development of the disease[(3)].

On the other hand, supplementation with natural compounds may represent an alternative strategy for prevention of different chronic pathologies. Of these natural supplements, vitamin D_3 and alpha-lipoic acid (ALA) have attracted our attention. Vitamin D, known for maintaining mineral homeostasis[(5)], was found to have other non-classic actions. It was engendered by the discovery of vitamin D_3 receptor in most of body tissues and cells[(6)], including pancreatic β-cells[(7)]. Vitamin D_3 was found to promote calcium absorption and utilization by β-cells, what is necessary for their function and insulin secretion[(7)]. In addition, the prevalence of cardiovascular risk factors, such as dyslipidemia, diabetes and obesity were increased in patients with low vitamin D concentrations[(8)]. Meanwhile, the stress hormone, cortisol, is known to decrease expression of vitamin D receptor [(9)]. Thus, the question arises to whether vitamin D_3 administration in stressed rats could modulate the expected metabolic changes. In addition, alpha-lipoic acid (ALA), a naturally occurring dithiol compound[(10)], is a powerful antioxidant and plays a fundamental role in the metabolism[(11)]. It targets cellular signal transduction pathways which increased glucose uptake and utilization, antagonizes the oxidative and inflammatory stresses[(12)], and improves glycemic control[(13)]. Also, ALA lowered blood lipids and was thought to be a possible protective agent against the risk factors of cardiovascular diseases[(14)].

With this background, the present study was conducted to evaluate the potential effects of natural supplementation by vitamin D_3 and alpha-lipoic acid on the metabolic changes induced in adult male rats subjected to chronic immobilization stress, as well as to probe the possible underlying mechanisms.

Materials and Methods:

The study was performed on 40 adult local strain albino male rats, initially weighing 200-300gm, purchased from a rat farm in Giza, and maintained in Medical Ain Shams Research Institute (MASRI) under standard conditions of boarding at room temperature, with normal light/dark cycle. Rats were given regular diet composed of bran bread, milk and vegetables (50-51% carbohydrates, 4-5% fats, 12-13% proteins and provided 300.19 Kcal/100 gm diet), with free access to water. Sample size was calculated using G power software. The study was approved by the Research Ethics Committee, Faculty of Medicine Ain Shams University (No. 0000175785). National Academies Press (NAP) Guide for the Care and Use of Laboratory Animals was followed[(15)].

Experimental groups:

Rats were randomly divided into the following groups (each group consists of 10 rats); **Control group:** Rats were maintained under the standard

conditions of boarding until sacrificed. **Stressed group**: Rats were separately subjected to immobilization stress by being encaged in tight restraining cages in the prone position 2 hours daily, 5 days/week, for 4 weeks[16]. **Vitamin D_3-treated stressed group**: Rats were subjected to immobilization stress and were treated at the same time by intraperitoneal (i.p.) injection of vitamin D_3 in a dose of 10 μg/Kg B.W., every other day[17]. **Alpha-Lipoic Acid (ALA)-treated stressed group**: Rats were subjected to immobilization stress and were treated at the same time by ALA in a dose of 50 mg/Kg B.W., given by gavage[18].

Chemicals and Drugs:

Vitamin D_3 was obtained as ampoules, 5mg/2ml each, supplied by CHEMIPHARM pharmaceuticals industries, Egypt. The contents of each ampoule were dissolved in coconut oil, supplied by Arab Perfumes, Chemicals and pharmaceutical Company (APC), Egypt, making final concentration of 1ml Vitamin D_3 /10 ml coconut oil.

Alpha- Lipoic acid was obtained as tablets (Thiotacid tablets), each containing 300 mg alpha-lipoic acid, supplied by EVA pharma for pharmaceutical and medical appliances SAE, Egypt. Tablets were ground and dissolved in normal saline solution to make a solution with lipoic acid concentration of 12.5 mg/0.1ml, and the solution was rendered alkaline (pH=7.8) by adding few drops of sodium hydroxide solution.

Experimental Procedures:

Initial body weight and body length were estimated at the beginning of the study. At the end of the experimental period (4 weeks), overnight fasted rats were weighed and anaesthetized by i.p. injection of thiopental sodium (Sigmatec Pharmaceutical industries, Egypt), in a dose of 40mg/Kg B.W., then body length was measured. And the following procedures were done:

- **Biochemical analysis:**

Blood samples were collected from abdominal aorta into heparinized tubes, that were centrifuged at 3000 rpm for 15 min. and the separated plasma were immediately used for determination of plasma glucose level according to Trinder[19] using kits supplied by Stanbio (USA), and the remained plasma was stored frozen at -80°C for later determination of lipid profile, plasma levels of malondialdehyde (MDA), total antioxidant capacity (TAC) and nitrite by colorimetric methods using kits supplied by Biodiagnostics (Egypt). Plasma insulin and leptin were measured by ELISA techniques using kits supplied by DRG instruments (USA). In addition, plasma testosterone and estradiol levels were measured by competitive immunoenzymatic colorimetric method using kits supplied by Novatec Immunodiagnostica GmbH (Germany).

- **Biological Studies:**

Immediately after collecting the blood samples, the diaphragm was exposed, quickly and carefully excised, and then was immediately placed in iced Krebs solution to be used for in vitro determination of glucose uptake as described by Saleh and Saleh[20]. Thereafter, both kidneys were dissected out, immediately placed in iced Krebs-Ringer solution to be used in determination of glucose output by the kidneys as described by Randall[21], with modifications of El-Nasr et al.[22]. Also, visceral fat was dissected out, dried with filter paper, and then weighed.

- **Histopathological Studies:**

Pancreas was dissected and preserved in 10% formalin. Small specimens were processed to form paraffin blocks for preparation of serial sections of 5µm thickness that were subjected to hematoxylin and eosin stain, Masson trichome stain for collagen fibers demonstration and Immunohistochemistry for Caspase-3 that was performed using avidin-biotin peroxidase technique using rabbit polyclonal antibody (anti Caspase-3 ab-4, rabbit polyclonal antibody, RB-1197-PO, Lot: 1197P10100. NeoMarkers, Fremont, California, USA) for detection of apoptotic cells. Sections were counterstained with Mayer's hematoxylin, then dehydrated, cleared, and mounted. The reaction appeared as brownish cytoplasmic granules with some nuclear staining. Negative control experiments were made by omitting the primary antibody. Palatine tonsil specimens were the positive control. All histological techniques were performed as described by Suvarna et al.[23].

Histomorphometrically, the area percentage (area%) of collagen fibers in Masson's trichome-stained sections and caspase-3 positive immunoreactive cells of islets of Langerhans of pancreas. Both were done using the image analyzer (Leica Q 500 MC program) in the Histology an Cell Biology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt. Five high power field/5 different sections in each rat were examined.

- **Anthropometric measures:**

The initial and final body weight [BW, (g)] and body length (naso-anal length) [length, (cm)] were used to determine initial and final body mass index (BMI) according to Nascimento et al.[24], as follows:

BMI = body weight (g)/length^2 (cm^2).

Then, BMI percentage change (**BMI%**) was calculated as follows:

BMI% =

(Final BMI-Initial BMI)/initial BMI X 100

High density lipoprotein-cholesterol (HDL-C) concentration (conc.) was calculated according to Friedewald et al.[25] from the formula:

HDL-C conc. (mg/dl) =

TC conc. - (LDL-C conc. + Tg conc. / 5).

Atherogenic index (AI) was calculated according to Malaspina et al.[26] from the formula:

Atherogenic index = TC conc. / HDL-C conc.

Both Homeostatic model assessment of β-cell function (HOMA-β) and insulin resistance (HOMA-IR) were calculated according to Matthews et al[27], as follows:

HOMA-B= 360 * [Fasting Insulin] / ([Fasting Glucose] - 63) %

HOMA-IR= Fasting insulin x Fasting glucose/ 405

Statistical analysis:

All statistical data and statistical significance were performed using IBM SPSS Statistics 20 (statistical program for social science) (version 20, SPSS Inc., Chicago, Illinois, USA). The one-sample Kolmogorov–Smirnov test was used to test for normality of variables. All variables were found to be normally distributed. Differences between groups were compared by one way ANOVA with least significant difference test (LSD) to find intergroupal significance. Final body mass index was compared with its initial value by Student's "t" test for paired data. The association

between the parameters was determined using the Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

Results:

As shown in table (1), there were insignificant differences in initial body mass index (IBMI) among all studied groups. Control and both treated stressed rats had significantly elevated final body mass index (FBMI) compared to their respective IBMI, while stressed rats exhibited insignificantly decreased FBMI compared to their IBMI. Stressed rats showed significantly lowered FBMI, BMI% and VFW when compared to the control rats. However, both treated stressed rat groups exhibited significant decrease in VFW compared to the control rats and significant increase in FBMI and BMI% as compared to stressed rats. Compared to both control and stressed rats.

Stressed rats showed significantly increased fasting plasma glucose and glucose output by kidneys, while the glucose uptake by diaphragm was significantly decreased. However, both treated groups showed significantly reduced fasting plasma glucose and glucose output by kidneys with significantly increased glucose uptake by diaphragm when compared with stressed rats, but all were normalized as compared with controls. Compared with Vitamin D_3 - treated stressed rats, ALA-treated stressed group showed non-significant changes in the 3 parameters as in (table 2).

Plasma triglycerides, total cholesterol, LDL-C and atherogenic index were all significantly increased in stressed group, while HDL-C was significantly decreased denoting dyslipidemic lipid profile. Both Vitamin D_3 and ALA- treated stressed groups showed significant reduction in Tg, TC, LDL-C and AI, but HDL-C was insignificantly changed when compared with stressed rats. Compared to the controls, both LDL-C, AI still significantly high, HDL-C still significantly low and Tg, TC were insignificantly changed in the treated groups. No significant changes were shown in the lipid profile among the treated groups (table 3).

As shown in table (4), plasma malondialdehyde (MDA) level was significantly increased while plasma total antioxidant capacity (TAC) showed significant decline in stressed rat group as compared with controls denoting occurrence of oxidative stress. Plasma insulin and testosterone were significantly decreased, while plasma estrogen was significantly increased in stressed rats as compared to controls. Both treated groups showed significant elevation in testosterone and significant reduction in estrogen when compared with stressed group. Only ALA-treated stressed group showed significant elevation in TAC and insulin when compared with stressed one. MDA and testosterone were significantly higher in both treated stressed groups compared to controls. In ALA-treated stressed group estrogen level still significantly higher than control rats. Compared to Vitamin D_3-treated stressed group, ALA-treated stressed group showed significant reduction in testosterone and significant elevation in estrogen level. However, both nitrite and leptin showed non-significant changes among all studied groups.

HOMA-B showed significant reduction in stressed rat group compared to controls denoting pancreatic B-cell affection. Both treated groups showed significantly increased HOMA-B when compared with stressed group being normalized when compared with controls. For ALA-treated group the HOMA-B was not significantly changed from Vitamin D3-treated stressed group. On the other hand, HOMA-IR showed non-significant change among the studied groups (table 5).

Table (1): Initial body mass index (IBMI, gm/cm), final body mass index (FBMI, gm/cm), body mass index percentage change (BMI, %) and visceral fat weight (VFW, gm) in the studied groups.

Experimental groups	IBMI	FBMI	BMI%	VFW
Control group	0.547 ± 0.015	0.627 ± 0.020	14.69 ± 2.203	2.87 ±0.380
P		**<0.001**		
Stressed group	0.536 ± 0.019	0.527 ± 0.019	-1.520 ± 1.374	1.12 ±0.310
P		**NS**		
P1	**NS**	**<0.001**	**<0.001**	**<0.001**
Vit. D_3-treated stressed group	0.546 ± 0.010	0.610 ± 0.016	13.330 ± 3.746	1.32 ±0.120
P		**<0.02**		
P1	**NS**	**NS**	**NS**	**<0.001**
P2	**NS**	**<0.002**	**<0.002**	**NS**
ALA-treated stressed group	0.551 ±0.015	0.599 ± 0.018	9.090 ± 3.270	1.08 ±0.160
P		**<0.05**		
P1	**NS**	**NS**	**NS**	**<0.001**
P2	**NS**	**<0.005**	**<0.02**	**NS**
P3	**NS**	**NS**	**NS**	**NS**

Values are expressed as mean ± SEM. **P**: Significance from initial value, calculated by Student's "t" test for paired data at P<0.05. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group. **NS:** Not significant.

Table (2): **Fasting plasma glucose level (mg/dl), glucose uptake by diaphragm (mg/100ml/g/90 min) and glucose output by kidneys (mg/g/60 min) in the studied groups.**

Experimental groups	Fasting Plasma glucose	Glucose uptake by diaphragm	Glucose output by kidneys
Control group	99.62 ±2.92	105.2 ±13.50	20.85 ±3.21
Stressed group	195.32 ±26.33	34.11 ±8.00	119.36 ±31.08
P1	**<0.001**	**<0.002**	**<0.001**
Vit. D_3-treated stressed group	100.18 ±6.68	71.90 ±16.42	13.92 ±1.67
P1	**NS**	**NS**	**NS**
P2	**<0.001**	**<0.05**	**<0.001**
ALA-treated stressed group	90.59 ±4.68	98.87 ±10.82	25.74 ±9.02
P1	**NS**	**NS**	**NS**
P2	**<0.001**	**<0.002**	**<0.001**
P3	**NS**	**NS**	**NS**

Values are expressed as mean ± SEM. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group. **NS:** Not significant.

Table (3): Plasma triglycerides (Tg), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and atherogenic index (AI) in the studied groups.

Experimental groups	Tg (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	AI
Control group	27.08 ±1.72	56.15 ±1.78	20.22 ±0.566	30.52 ±1.87	1.88 ±0.069
Stressed group	45.78 ±3.38	101.69 ±8.92	62.92 ±7.09	20.82 ±2.51	4.72 ±0.503
P1	**<0.001**	**<0.001**	**<0.001**	**<0.005**	**<0.001**
Vit. D_3-treated stressed group	27.69 ±2.04	68.17 ±4.50	38.66 ±4.07	23.24 ±1.67	2.92 ±0.239
P1	**NS**	**NS**	**<0.01**	**<0.02**	**<0.01**
P2	**<0.001**	**<0.001**	**<0.002**	**NS**	**<0.001**
ALA-treated stressed group	27.88 ±1.92	79.50 ±5.90	49.41 ±4.42	23.39 ±2.00	3.30 ±0.197
P1	**NS**	**<0.002**	**<0.001**	**<0.02**	**<0.001**
P2	**<0.001**	**<0.002**	**<0.05**	**NS**	**<0.002**
P3	**NS**	**NS**	**NS**	**NS**	**NS**

Values are expressed as mean ± SEM. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group. **NS:** Not significant.

Table (4): Plasma malondialdehyde (MDA), total antioxidant capacity (TAC), nitrite, insulin, leptin, testosterone, and estrogen in the studied groups.

Experimental groups	MDA (nmol/ml)	TAC (mM/l)	Nitrite (µmol /l)	Insulin (µlU/ml)	Leptin (pg/ml)	Testosterone (pg/ml)	Estrogen (pg/ml)
Control group	2.12 ±0.076	7.14 ±0.1	33.61 ±1.97	13.15 ±2.19	0.132 ±0.034	3.81 ±0.457	18.17 ±1.40
Stressed group	4.14 ±0.433	5.67 ±0.430	37.24 ±2.61	7.91 ±1.52	0.106 ±0.024	0.865 ±0.074	71.59 ±4.3
P1	**<0.001**	**<0.002**	**NS**	**<0.05**	**NS**	**<0.001**	**<0.001**
Vit. D_3-treated stressed group	3.60 ±0.179	6.44 ±0.31	37.2 ±2.65	11.22 ±0.963	0.077 ±0.016	16.11 ±0.088	18.43 ±1.68
P1	**<0.001**	**NS**	**NS**	**NS**	**NS**	**<0.001**	**NS**
P2	**NS**	**NS**	**NS**	**NS**	**NS**	**<0.001**	**<0.001**
ALA-treated stressed group	3.43 ±0.125	6.74 ±0.206	35.96 ±2.04	12.73 ±1.28	0.152 ±0.047	12.57 ±0.591	47.46 ±4.34
P1	**<0.002**	**NS**	**NS**	**NS**	**NS**	**<0.001**	**<0.001**
P2	**NS**	**<0.02**	**NS**	**<0.05**	**NS**	**<0.001**	**<0.001**
P3	**NS**	**NS**	**NS**	**NS**	**NS**	**<0.001**	**<0.001**

Values are expressed as mean ± SEM. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group.
NS: Not significant.

Table (5): Homeostatic model assessment of beta cell function (HOMA-B) and homeostatic model assessment of insulin resistance (HOMA- IR) in the studied groups.

Experimental groups	HOMA-B	HOMA-IR
Control group	138.99 ±28.18	3.23 ±0.548
Stressed group	33.63 ±10.04	3.09 ±0.761
P1	**<0.01**	**NS**
Vit. D_3-treated stressed group	131.7 ±31.76	2.9 ±0.349
P1	**NS**	**NS**
P2	**<0.02**	**NS**
ALA-treated stressed group	183.57 ±30.82	2.87 ±0.468
P1	**NS**	**NS**
P2	**<0.002**	**NS**
P3	**NS**	**NS**

Values are expressed as mean ± SEM. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group. **NS:** Not significant.

Histopathological study of pancreatic tissue:

A) Hematoxylin & Eosin (H&E):

Examination of H&E-stained sections of pancreas of control rats showed the pancreatic lobules containing endocrine and exocrine components. The islets of Langerhans representing the endocrine part, was seen composed of rounded cells with pale-stained cytoplasm and central rounded vesicular nuclei. Blood sinusoids were scattered in between the islets' cells. The exocrine part consisted of pancreatic acini, formed of collection of pancreatic acinar cells having basal basophilic cytoplasm, basal rounded vesicular nuclei and apical acidophilic zymogen granules (figure 1A). The pancreas of stressed rats showed cytoplasmic vacuolizations in many cells inside the islets with karyolitic and pyknotic nuclei were seen in many cells (figure 1B). Vitamin D_3-treated stressed rats showed apparently no improvement compared to stressed rats. Few cells of the islets of Langerhans were seen with vacuolated cytoplasm. Some peripheral cells contained with pyknotic

nuclei. Dilated blood sinusoids were also noticed (figure 1C). Alpha-lipoic acid-treated stressed rats showed evident improvement of the histological picture of the islets of Langerhans compared to stressed rats. Most of the cells of the islets of Langerhans were comparable to the control group. Few peripherally situated cells were seen with vacuolated cytoplasm and pyknotic nuclei (figure 1D).

B) Masson trichrome stain:

Masson's trichrome-stained pancreas sections of control rats showed minimal collagen fibers inbetween cells of the islets of Langerhans and around the pancreatic acini (figure 2A). Stressed (figure 2B), Vitamin D3-treated stressed (figure 2C) and alpha-lipoic acid stressed (figure 2D) rats were comparable to control group. Table 6 shows that area % of Collagen fibers was insignificantly changed among the 4 studied groups.

C) Caspase 3 immunostaining:

Caspase 3 immunostaining of pancreas of control rats, showed minimal caspase 3 immunoreactivity in the islets of Langerhans and in pancreatic acinar cells (figure 3A). Stressed rats showed numerous caspase 3 immunopositive islet cells and pancreatic acinar cells (figure 3B). Vitamin D_3-treated stressed rats showed minimal caspase 3 immunoreactivity (figure 3C). Alpha-lipoic acid treatment revealed little caspase 3 immunoreactivity (figure 3D). Area % of Caspase-3 immunopositive cells in islets of Langerhans was significantly increased by stress as compared to control group. This area % was significantly decreased by the two treating models when compared with stressed group being non significantly changed from controls as shown in table 6.

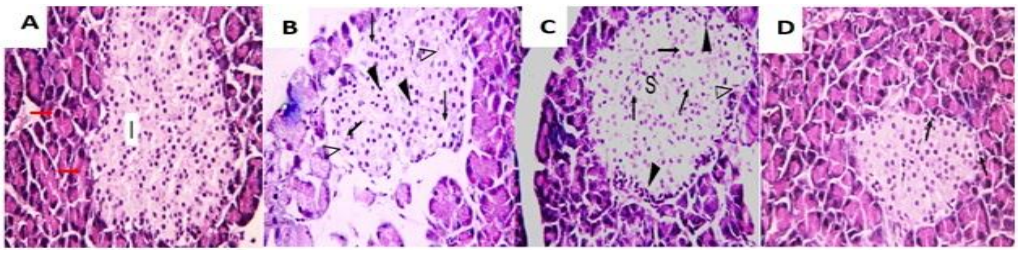

Figure (1): Photomicrographs showing structure of rat pancreas in the different studied groups (H&E x 400). **(A)** Control group showing normal islets of Langerhans (I) and pancreatic acini (red ↑). **(B)** Stressed group showing cells of the islets having vacuolated cytoplasm (↑), Karyolitic (Δ) or pyknotic (▲) nuclei. **(C)** Vitamin D_3-treated stressed group showing few islet cells with vacuolated cytoplasm (↑). Notice peripheral cells containing pyknotic nuclei (▲). Dilated blood sinusoids can be observed (S). **(D)** Alpha-Lipoic acid-treated stressed group showing improvement of pancreatic structure. Few peripherally situated cells can be seen with vacuolated cytoplasm and pyknotic nuclei (↑).

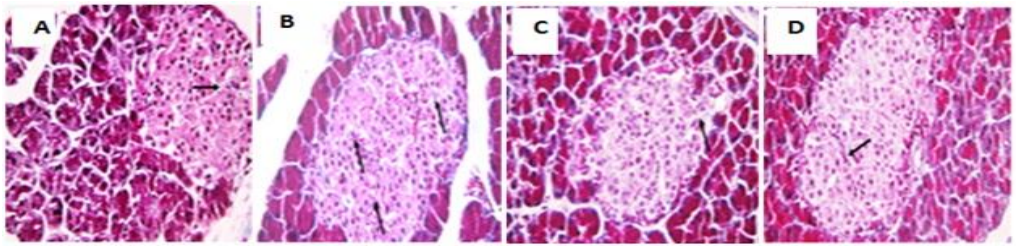

Figure (2): Photomicrographs of rat pancreas showing collagen fibers content around islets of Langerhans and in-between the pancreatic acini of the different studied groups (Masson's trichrome stain x 400**). (A)** Control group showing minimal collagen content (↑). **(B)** Stressed group showing minimal collagen fibers (↑) **(C)** Vitamin D_3-treated stressed group showing minimal collagen fibers (↑). **(D)** Alpha-Lipoic acid-treated stressed group showing minimal collagen fibers (↑).

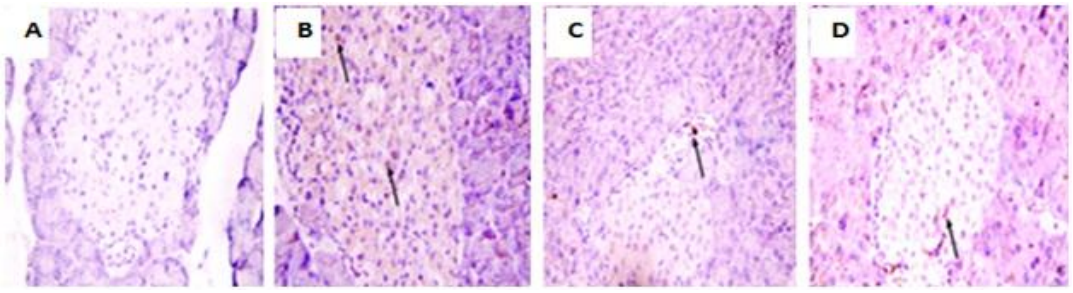

Figure (3): Photomicrographs of rat pancreas showing caspase 3 immunoreactivity in cells of islets of Langerhans and in pancreatic acinar cells of different groups (Avidine-Biotin immune-peroxidase technique x 400**). (A)** Control group showing minimal caspase 3 immunoreactive cells (↑). **(B)** Stressed group showing numerous caspase 3 immunoreactive cells (↑) **(C)** Vitamin D_3-treated stressed group showing minimal caspase 3 immunoreactive cells (↑). **(D)** Alpha-Lipoic acid-treated stressed group showing minimal caspase 3 immunoreactive cells (↑).

Table (6): Area % of collagen fibers and caspase-3 immunopositive cells in islets of Langerhans in the studied groups.

Experimental groups	Collagen fibers area %	Caspase 3 area %
Control group	0.23 ±0.009	0.29 ±0.010
Stressed group	0.29 ±0.005	2.23 ±0.063
P1	**NS**	**<0.001**
Vit. D_3-treated stressed group	0.22 ±0.007	0.33 ±0.004
P1	**NS**	**NS**
P2	**NS**	**<0.001**
ALA-treated stressed group	0.25 ±0.007	0.63 ±0.013
P1	**NS**	**NS**
P2	**NS**	**<0.001**
P3	**NS**	**NS**

Values are expressed as mean ± SEM. **P_1**: Significance by LSD at P<0.05 from control group. **P_2**: Significance by LSD at P<0.05 from stressed group. **P_3**: Significance by LSD at P<0.05 from Vit.D_3-treated stressed group. **NS:** Not significant.

Correlation study:

A correlation study for plasma insulin level revealed a positive correlation with glucose uptake by diaphragm (r= 0.408, P<0.02), and negative correlation with glucose output by kidneys (r= -0.378, P<0.05).

Meanwhile, plasma MDA level has significant positive correlations plasma Tg level (r= 0.365, P<0.05), LDL-C level (r= 0.399, P<0.05) and AI (r= 0.423, P<0.02), however, it has a significant negative correlation with VFW (r= -0.541, P<0.002).

Plasma TAC level has a significant positive correlation with glucose uptake by diaphragm (r= 0.369, P<0.05), HOMA-B (r= 0.351, P<0.05), although it has significant negative correlations with fasting plasma glucose level (r= -0.447, P<0.01), glucose output by kidneys (r= -0.369, P<0.02), plasma Tg (r= -0.413, P<0.01), TC (r= -0.588, P<0.001), LDL- C (r= -0.607, P<0.001) and AI (r= -0.557, P<0.002).

Plasma testosterone level has significant negative correlations with fasting plasma glucose level (r= -0.470, P<0.001), glucose output by kidneys (r= -0.465, P<0.005) and plasma Tg (r= -0.482, P<0.005).

Plasma estrogen has significant positive correlations with fasting plasma glucose, glucose

output by kidney, plasma Tg, TC, LDL- C and AI (r= 0.616, o.623, 0.592, 0.594, 0.691 and 0.691 respectively, all at P<0.001). Also, estrogen is found to be negatively correlated to glucose uptake by diaphragm (r= -0.383, P<0.02) and HOMA-B (r= -0.363, P<0.05).

Discussion:

To the knowledge of the authors, this is the first study to deal with the effect of natural supplementation by Vitamin D3 and Alpha-Lipoic Acid on glucose and lipid metabolic dysfunction in face of chronic immobilization stress in adult male rats. Metabolic effect of chronic immobilization stress was previously discussed in a work by the authors[28].

Vitamin D_3-treated stressed rats showed a significant increase in FBMI and *BMI%* compared to the stressed group and significant reduction of *VFW* compared to the controls which is indicative for the increased lean muscle mass effect of vitamin D_3. Similarly, Fornari et al.[29] demonstrated that vitamin D_3 is associated with higher levels of muscle mass in male obese individuals, suggesting a positive protective role of vitamin D_3 on both muscle mass and strength. Remelli et al. reviewed the biological, clinical and epidemiological evidence supporting the association between vitamin D deficiency and an increased risk of sarcopenia in older people[30] On the contrary, Sadiya et al.[31] found no effect of long term-vitamin D_3 supplementation on weight, fat mass or waist circumference in type 2 diabetic obese vitamin D_3 deficient subjects. The testosterone rise and the estrogen decline in vitamin D_3 treated stressed group, herein, could explain the rise of BMI%, although reducing adiposity, through elevating the fat free body weight. In support, Tirabassi et al.[32] demonstrated that testosterone decline could cause a lack of negative regulation of both of the differentiation of pre-adipocytes into mature adipocytes, and of the differentiation of mesenchymal stem cells to adipocytes through the androgen receptor expressed mainly in visceral fat. Moreover, the associated oxidative stress indicated by the elevated MDA level could explain the reduction in VFW, this is supported by the negative correlation observed between MDA and VFW.

Regarding the *glycemic control* in vitamin D_3 treated stressed rats, amelioration of immobilization stress-induced hyperglycemia, hypoinsulinemia, reduced diaphragmatic glucose uptake and elevated renal glucose output was observed. These improvements may be due to the rise of insulin release in these rats, as shown by increased plasma insulin level to reach control values. In support, HOMA-B was normalized in this group indicating significant improvement in the β-pancreatic cells function, which is, also, evidenced by the histological picture of the pancreas that showed significantly decreased area % of caspase-3 compared to stressed group, denoting less apoptosis in pancreatic tissues, with a significant positive correlation between insulin and glucose uptake by diaphragm and negative correlation between insulin and glucose output by the kidney. This vitamin D_3-induced better glycemic control is in agreement with Segovia-Ortí et al.[33]. The vitamin D_3 preserving effects on pancreatic structure, observed herein, could be supported by the study of Kayaniyil et al.[34] which showed that vitamin D_3 improved insulin synthesis and secretion due to the presence of vitamin D receptor (VDR) on the pancreatic β islet cells. In

these cells, the biologically active metabolite of vitamin D_3 stimulates insulin production and secretion by binding with VDR[35]. Also, Riachy et al.[36] reported that vitamin D_3 is a β cell protector against cytokine-induced apoptosis. Thus, the normalizing effects of vitamin D_3 in stressed rats on glycemic parameters and pancreatic structure could be, also, mediated by its anti-apoptotic effects. Another mechanism which is proposed to contribute in the beneficial role of vitamin D_3 against hyperglycemia induced by stress could be increased testosterone and reduced estrogen level by vitamin D_3 and this is supported, herein, by the negative correlation between testosterone and each of fasting plasma glucose level and glucose output by the kidney, as well as the positive correlation between estrogen and each of fasting plasma glucose level and glucose output by the kidney and the negative correlation between estrogen and each of glucose uptake by diaphragm and HOMA-B.

In the current study, vitamin D_3-treated stressed rats exhibited improvement of stress-induced *dyslipidemia*, similar to Bonakdaran et al.[37] and Zittermann et al.[38]. However, Jorde et al.[39] found that high dose-vitamin D_3 supplementation in diabetic patients did not improve their abnormal lipid profile. The vitamin D_3 improving effects on lipid changes in stressed rats, herein, could be explained by many mechanisms. According to Zittermann et al.[38], vitamin D_3 could have reduced serum triglycerides directly and indirectly by affecting serum parathyroid hormone (PTH) and/or the calcium balance. In addition, Eftekhari et al.[40] suggested that the vitamin D_3-induced calcium rise could lower serum triglycerides by reducing hepatic triglyceride formation and secretion. Also, Rodriguez et al.[41] suggested that the reduced serum PTH may reduce serum triglycerides via an increase in its peripheral uptake. Moreover, vitamin D_3 was reported to induce the expression of VLDL-cholesterol gene receptors in some cell types[42]. In addition, the elevation of plasma testosterone level by vitamin D_3 administration in stressed rats could be implicated in the reduction of plasma lipids, TC, Tg, LDL- C as well as AI. The significant negative correlation between plasma testosterone and plasma Tg supports this view. Further, vitamin D_3 partially limited the rise of plasma LDL-C level and AI, which were still higher than control values in these rats. These varied effects are assumed to be related to the persistent oxidative stress in vitamin D_3 treated stressed rats, denoted by the higher MDA. This explanation could be supported by the significant positive correlations between MDA and plasma Tg, LDL-C and AI observed in this study. Also, the plasma TAC was found to be negatively correlated with plasma lipids.

Regarding the *oxidative stress*, vitamin D_3-treated stressed rats displayed a tendency towards partial improvement of the oxidative state in agreement with Saif-Elnasr et al.[43]. The improvement of the antioxidant defense could be a suggested mechanism for the beneficial effect of vitamin D_3 on stress- mediated dyslipidemia and hyperglycemia.

Regarding the *hormonal changes*, vitamin D_3 treatment, herein, was found to normalize sex hormone levels in stressed rats. The restoration of the normal hormonal pattern in these rats, is in agreement with Pilz et al.[44] & Nimptsch et al.[45] & Zhao et al.[46]. On the contrary, Jorde et al.[47] reported that vitamin D_3 supplementation in adult males showed absence of testosterone rise. This

significant rise of testosterone could be attributed to the direct effect of vitamin D_3 on testosterone secretion. This explanation could be supported by studies of Blomberg et al.[48] and Blomberg[49]. These studies demonstrated that VDR and vitamin D_3-metabolizing enzymes were present in the testes. In support, Hofer et al.[50] suggested that vitamin D_3 had a major role in male steroidogenesis. In addition, the testosterone rising effect of vitamin D_3, herein, could be mediated by the ability of vitamin D_3 to bind to androgen receptors. In accordance, Proal et al.[51] demonstrated that active vitamin D_3 has high affinity for some of the body's other nuclear receptors, rather than activating the VDR. The study suggested that vitamin D_3 when being elevated above its normal range, could bind to thyroid receptors, the glucocorticoid receptors and the androgen receptor, displacing their native ligands. Furthermore, the observed vitamin D_3 correction of the hormonal disturbance that occurred in stressed rats, is similar to Canguven et al.[52]. These effects could be due to tissue-specific effects of vitamin D_3 on estrogen and androgen production/metabolism[53]. This estrogen lowering effect of vitamin D_3 could, also, explain the associated improvement of dyslipidemia in these stressed rats. In support, there were significant positive correlations between estrogen and plasma lipids, namely Tg, TC, LDL-C and AI.

Upon ***Alpha-Lipoic acid*** supplementation to stressed rats, *BMI%* was significantly elevated to approach control values, which indicates that ALA counteracted the effect of stress on BMI%. Meanwhile, the *VFW* was still significantly lower than the control rats, a finding that could be explained by the ability of lipoic acid to prevent fat accumulation[54]. Thus, the increased BMI% is suggested to be due to increase of muscle mass and not due to increase of fat mass. The increased level of plasma testosterone in lipoic acid treated rats could be responsible for such effect due to its known anabolic role.

Regarding the *glycemic parameters*, stressed rats given lipoic acid displayed an improved stress-induced glycemic state, which is in consistence with El-Midaoui and de Champlain[55] and Winiarska et al.[56]. The higher glucose uptake by diaphragm induced by lipoic acid is in accordance to Moini et al.[57], who found that lipoic acid restored insulin-stimulated glucose uptake into skeletal muscles. Similarly, Saengsirisuwan et al.[58] found that postprandial glucose was attenuated with lipoic acid administration, indicating higher skeletal muscle glucose transport activity. On the contrary, no significant changes in fasting plasma glucose levels were reported by Jacob et al.[59] after intravenous administration of lipoic acid daily for 10 days in type 2 diabetic patients. The increased plasma insulin level observed following lipoic acid treatment in stressed rats disagrees with Amirkhizi et al.[60]. They found that lipoic acid supplementation for 12 weeks caused lowering of serum insulin levels compared to the placebo group, with no change in fasting glucose level. Also, de Oliveira et al.[61] found that lipoic acid did not affect serum insulin levels. This discrepancy may be due to varied methodologies used in these studies, and due to the different physiological mechanisms between animals and human in insulin metabolism. The glycemic improvements observed upon lipoic acid treatment to stressed rats in the current study could be explained by increased insulin secretion in this

group. The increased insulin contributed to the glucose lowering effect of lipoic acid via increasing the glucose uptake by the diaphragm as observed herein. In addition, restoration of pancreatic B-cell function, is also, evidenced by the improved histological picture of the pancreas. Lipoic acid administration was able to cause normalization of pancreatic structure. These data denote the ability of lipoic acid to counteract apoptosis in pancreatic tissue that was induced by immobilization stress, In support, Poh and Goh[(62)] demonstrated that chronic lipoic acid treatment elevated both insulin stimulated glucose oxidation and glycogen synthesis. Further, lipoic acid-induced hyperinsulinemia is suggested to be mediated by affecting the insulin signaling pathway, such as increase in phosphatidylinositol-4, 5-biphosphate 3-kinase (PI3-kinase) and protein kinase B as reported by Konrad et al.[(63)]. Moreover, lipoic acid mediated direct mechanisms which are insulin-independent could, also, be involved in the beneficial glycemic effect of lipoic acid such as increased glucose entry into cells, increased glucose consumption and decrease gluconeogenesis as observed in the current study. In support, lipoic acid was found to stimulate glucose uptake [(64)], via stimulating translocation of the GLUT1 (insulin-independent) and GLUT4 (insulin dependent). In addition, lipoic acid could increase GLUT4 translocation to the plasma membrane independent of insulin, via enhancing the expression of the GLUT4 gene through stimulating binding of the transcription factor myocyte enhancer factor-2 (MEF-2) to promoters in the GLUT4 gene[(63)].

Lipoic acid administration to stressed group revealed improvement of immobilization stress-induced *dyslipidemia* in accordance to Zhang et al.[(65)] and Mohammadi et al. [(66)]. Also, Valdecantos et al.[(67)] demonstrated that alpha lipoic acid supplementation in normal healthy Wistar rats for 8 weeks directly decreased plasma Tg, free fatty acids and ketone bodies levels. In the current study, lipoic acid treatment exerted a beneficial effect on all components of lipid profile; however, HDL-C was unchanged when compared to stressed rats. These findings are in agreement with the study of Mousavi et al.[(68)]. On the other hand, the lipid lowering effects of lipoic acid in stressed rats disagree with many previous studies. In a short-term clinical trial, lipoic acid supplementation in a dose of 400 mg/day for 4 weeks had no effect on serum TC and Tg in type 2 diabetic patients, while HDL-C concentration was significantly improved[(69)]. The triglycerides lowering effects of lipoic acid, observed herein, could be due suppression of lipogenesis by down-regulation of hepatic acetyl- CoA carboxylase and fatty acid synthase expression, and increasing fat oxidation in liver by enhancing carnitine palmitoyl transferase Ia expression as well as enhancing the activities of lipoprotein lipase and lecithin cholesterol acyl transferase (LCAT)[(70)]. Meanwhile, the cholesterol lowering effects of lipoic acid could be mediated by elevating hepatic LDL receptor protein[(71)]. Despite the improvement in lipid levels and normalization of Tg in stressed rats following lipoic acid administration herein, TC, LDL-C, and AI were still elevated, HDL-C was reduced when compared to the control ones. This effect could suggest that the beneficial effect offered by lipoic acid against the stress-induced dyslipidemia, did not reach the full picture of normalization, an effect that could be attributed to

the use, in this study, of different lipoic acid dose according to the studies of Moura et al.[72] and Mousavi et al. [68]. Also, these variable effects on lipid profile are suggested to be attributed to the incomplete correction of oxidative state in lipoic acid treated stressed rats. Such assumption, could, also, be supported by significant negative correlations of plasma TAC and plasma lipids in this study. In support, lipoic acid was found to have blood lipid modulating effects through its antioxidant and anti-inflammatory features[73,74]. Thus, the antioxidant property of lipoic acid was suggested to induce many beneficial effects on regulating lipid metabolism abnormalities due to lipoic acid potency in preventing LDL oxidation[75]. Oxidized LDL, derived from LDL-C under oxidative stress, has proinflammatory and prothrombotic effects[76].

Regarding the *oxidative stress* markers, lipoic acid treatment in stressed rats elevated plasma TAC, although it did not affect plasma MDA or nitrite level compared to stressed ones. This unpredicted effect could be attributed to the accompanied sex hormonal changes induced by immobilization stress and/or the used dose or duration of lipoic acid administration in this study. The current antioxidant effect of lipoic acid could be explained according to Jones et al.[77]. They reported that both the oxidized and reduced forms of ALA are powerful antioxidants whose functions include: (1) quenching of ROS, (2) regeneration of exogenous and endogenous antioxidants such as vitamins C and E and reduced glutathione (GSH), (3) chelation of metal ions, (4) reparation of oxidized proteins (5) regulation of gene transcription, and (6) inhibition of the activation of nuclear factor kapp B (NF-κB). Moreover, ALA was found to be one of the inducers of nuclear factor erythroid 2- related factor 2 (Nrf-2) which mediates the expression of antioxidant gene and significantly increased cellular capacity of GSH synthesis[78]. Busse et al. [79] reported that ALA, also, increases GSH levels through its ability to increase cysteine uptake.

Regarding the *sex hormonal changes*, lipoic acid treatment elevated plasma testosterone level accompanied by reduced plasma estrogen level compared to stressed rats. These findings are similar to Othman et al.[80]. These effects were mediated by defending against oxidative stress, and by protecting testosterone synthesis pathway across hypothalamus-testicular axis. This reversal of stress-induced sex hormones alteration could be an important mechanistic tool of lipoic acid to antagonize immobilization stress dyslipidemic effects. However, the incomplete restoration of plasma estrogen levels in the current work, to reach control values, could provide explanation for the incomplete correction of the dyslipidemic effects in lipoic acid. This suggestion could be supported by the significant positive correlations of plasma estrogen and plasma lipids, namely Tg, TC, LDL-C and AI. However, the significant testosterone rising effect in lipoic acid treated males compared to either control rats or stressed males disagree with Lebda et al. [81]. They demonstrated that lipoic acid supplementation alone in adult males caused no effect on testosterone level. The higher plasma testosterone level induced by lipoic acid treatment in stressed males, herein, compared to either control rats or stressed ones, could be attributed to its anti-inflammatory and antioxidants effects according to the study by Pinar et al.[82]. Also, Prathima et al.[83]

observed that lipoic acid was a potent antioxidant, alleviating carbimazole-induced testicular oxidative stress in adult rats. They found that testicular preserving effects of lipoic acid were mediated by improving the testicular steroidogenesis and spermatogenesis, reducing the testicular damage and elevating the testicular activity levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and GSH content. Therefore, the aforementioned lipid lowering effects and improved glycemic parameters by lipoic acid administration, herein, could be attributed to both improved TAC and resuming the male sex hormonal pattern mediated by lipoic acid supplementation in stressed rats.

It is worth noting that in stressed rats of the current study, lipoic acid was less efficient than vitamin D_3 in combating stress-induced alteration in estrogen hormone. This observation was manifested as significantly higher plasma estrogen in lipoic acid treated stressed males compared to vitamin D_3 treated stressed males in this study in which estrogen level approached control value. Also, it is of value to demonstrate in the current study, for the first time, the effects of lipoic acid on sex hormones changes in immobilization stress.

Conclusion:

Both vitamin D_3 and alpha lipoic acid supplementation to stressed male rats proved to be effective in amelioration of the glycemic and lipidemic derangements induced by exposure to chronic immobilization stress. Such beneficial effect of both agents could be attributed to their direct effects, their successful role in restoration of the normal sex hormone pattern, their antioxidant potential as well as antiapoptotic effect.

Limitations and Recommendations:

Based on the abovementioned benefits of administration of the current regimen of vitamin D_3 and alpha lipoic acid in males as preventive agents of value in abating metabolic hazards resulting from stress. We recommend trial of different doses and durations of both treatments on other forms of stress either acute or chronic and to include females in the study.

Conflict of interest:

The authors declare that there is no conflict of interest associated with this work.

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