

Thyroid Function Changes in a Rat Model of Vitamin D Deficiency and Effect of Vitamin D and Metformin Treatment

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Abstract

Background: Previous studies showed controversies about the association of vitamin D deficiency and thyroid dysfunction and up to our knowledge there was no study that were conducted to study the effect of vitamin D deficiency on thyroid function. Metformin is an antidiabetic drug that is widely used and also there was contradictory data about its effect on thyroid function.

Aim of Study: To identify whether vitamin D deficiency is considered a cause for hypothyroidism or not. Also, to investigate the effect of metformin treatment on thyroid function in a rat model of vitamin D deficiency.

Material and Methods: 40 Adult male albino rats of local strains were divided randomly into 5 equal groups; control, control metformin treated (control + metformin), vitamin D deficient, vitamin D deficient metformin treated (vitamin D deficient + metformin) and vitamin D deficient vitamin D treated (vitamin D deficient + vitamin D).

Results: Vitamin D deficiency model significantly increased final Body Mass Index (BMI), relative thyroid weight, serum Thyroid Stimulating Hormone (TSH), serum reverse triiodothyronine (rT3) and hepatic deiodinase 3 activity but significantly declined serum levels of each of triiodothyronine (T3), thyroxine (T4), 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)2D) when compared to control group. Metformin treatment of vitamin D deficient rats significantly reduced relative thyroid weight with a significant decreased in TSH level when compared with vitamin D deficient group. A significant negative correlation was reported between serum 25(OH)D and each of final BMI, serum TSH, relative thyroid weight, hepatic deiodinase activity and serum rT3 but it was positively correlated to serum level of both T3 and T4. Histopathology and histomorphometric studies of the thyroid gland in vitamin D deficient rats showed a large number of follicles with increased dimensions, the follicular colloid showed no vacuolation, the amount of colloid increased and the follicles were found to be lined by simple cuboidal epithelial cells with basal nuclei.

Conclusion: As vitamin D deficiency decreased thyroid function through affecting hepatic deiodinase 3 activity and this change was deteriorated with vitamin D supplementation,

thus, monitoring of vitamin D levels and its supplementation may play a role in management of hypothyroidism. Also, metformin exerted an anti-proliferative activity, improved thyroid structure and lowered TSH without affecting serum levels of T4 or T3, thus it could provide a rationale for an innovative therapy of thyroid proliferative diseases.

Key Words: *Thyroid function – Vitamin D deficiency – Metformin – Rat.*

Introduction

THYROID gland synthesizes, stores and releases thyroid hormones, thyroxine (T4) and triiodothyronine (T3), which are essential for maintenance of an appropriate function of all tissue and cell types. Their synthesis is stimulated by the Thyroid Stimulating Hormone (TSH) released by the anterior pituitary [1]. T4 constitutes approximately 85% of both hormones' secretion and is produced only by the thyroid gland, while T3 is mainly formed by deiodination of T4 in peripheral tissues and has a 3-to 8-fold greater biological activity compared to T4 which is considered a prohormone [2]. Metformin is a worldwide used anti-diabetic drug [3]. Also, it was reported that metformin can be used as an antiaging agent, an anticancer agent, a neuroprotective agent, a cardiovascular protective agent and an optional drug for polycystic ovary syndrome [4]. Interestingly, some studies showed that metformin would affect the levels of TSH without relevant changes in serum T4 and T3 levels or clinical symptoms of hyperthyroidism [5,6], with or without thyroid dysfunction [7]. On the other hand, Rotondi, et al. [8], Díez and Iglesias [9] and Reddy, et al. [10] found that treatment with metformin in euthyroid patients was not associated with significant changes in the serum levels of TSH. Vitamin D encompasses several secosteroid compounds; 2 of them, cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), are commonly

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referred to with this name [11]. The former is synthesized in the skin upon exposure to ultraviolet B radiation by 7-dehydrocholesterol reductase and acquired from few dietary sources (mainly fatty fish), while the latter is synthesized by plants and fungi, which may constitute vitamin D₂ dietary source for humans [11]. Both vitamin D₂ and D₃ are hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D, calcidiol), which is the major circulating and storage form of vitamin D. It has little biological activity, however, its serum concentration is universally acknowledged to reflect vitamin D status [12]. The active hormone is acquired by hydroxylation of 25(OH)D to 1,25 – dihydroxyvitamin D (1,25(OH)₂D, calcitriol). This conversion takes place mainly in the kidney. It has been proposed that serum calcidiol level serves as the main determining factor of extrarenal calcitriol synthesis [13]. Serum 25(OH)D is used to reflect vitamin D status because it is the major circulating precursor of active vitamin D and has a half-life, ranging from two to three weeks [14]. Interestingly, 1, 25-(OH)₂D has a short circulating half-life and is tightly under the influence of parathyroid hormone, calcium and phosphate. Moreover, low level of serum 1, 25-(OH)₂D may not be observed until vitamin D deficiency is severe [15]. Vitamin D deficiency is a worldwide problem [16]. It has been estimated that more than one billion people in the world have vitamin D deficiency [17]. The high incidence of obesity in the population is a determinant of vitamin D deficiency, as circulating levels are lower due to dilution in the fat mass [18]. Vitamin D deficiency was associated with an increased risk of hypertension, cardiovascular disease, infectious and autoimmune diseases, glucose intolerance, albuminuria, and cancer [19]. Mackawy, et al. [20], Aljohani, et al. [21] and Idiculla, et al. [22] reported low serum levels of vitamin D in hypothyroid patients. On the other hand, Goswami, et al. [23] and Musa, et al. [14] declared a non-significant difference in the level of vitamin D among the women with hypothyroid compared to the control. Alrefaie and Awad [24] found that vitamin D increased deiodinase 2 expression and hence the peripheral conversion of T₄ into T₃. But almost all previous studies evaluated the association between vitamin D level changes and thyroid disorders and up to our knowledge no study assessed the effect of vitamin D deficiency on thyroid function. Also, limited data was reported about effect of metformin treatment on thyroid function in vitamin D deficient rats. Thus, this study aimed to clarify the effect of vitamin D deficiency on thyroid structure and function and the possible mechanism involved. Also, to identify the effects of metformin

treatment on thyroid function and structure changes in vitamin D deficient rats.

Material and Methods

In the period between April 2019 and July 2019, this study was achieved in Scientific and Medical Research Center (ZSMRC) in Faculty of Medicine, Zagazig University. 40 Adult male albino rats of local strains weighing 150-190g, obtained from Zagazig Faculty of Veterinary Medicine, housed 4/cage in a light-and temperature-controlled room on a 12h/12 h light-dark cycle and fed a standard pellet lab chow with ad libitum access to tap water [25]. The experimental protocol in this study was conducted according to the data guiding the use of research animals and was approved by the Institutional Animal Care and Use Committee (IACUC), Zagazig University.

Experimental design:

After one week of accommodation, rats were divided equally into 5 groups with 8 rats each; control, control and metformin treated (control + metformin), vitamin D deficient, vitamin D deficient and metformin treated (vitamin D deficient + metformin), and, vitamin D deficient and vitamin D treated (vitamin D deficient + vitamin D). In both control and control + metformin groups, rats fed ordinary diet, while, rats in the vitamin D deficient groups were fed a vitamin D deficient diet (TD.87095, Harlan Laboratories, Madison, WI, USA) containing 1.25% phosphate, 2% calcium and 20% lactose [18]. The high phosphate, calcium and lactose in vitamin D deficient diet was considered to maintain normal intestinal phosphate and calcium absorption and consequently normal serum parathyroid hormone [26]. Also, the rats in vitamin D deficient groups received intraperitoneal (i.p.) injections of 32ng of 19-nor-1,25-dihydroxyvitamin D₂ (paricalcitol; Zemplar, AbbVie, Berkshire, UK) on days 1, 3, 5, 8, 10, and 12 [18]. Paricalcitol, calcitriol analogue, was used to induce expression of renal cytochrome P450 family 24 subfamily A member 1 (CYP24A1), the enzyme responsible for the degradation of endogenous 25(OH)D and calcitriol [18]. This rat model, within three weeks, should induce deficiency in both 25(OH)D and calcitriol [18]. Thus, blood samples were drawn, from tail vein, at day 22 and serum samples were analyzed for 25(OH)D to confirm hypovitaminosis D and rats which did not achieve decreased 25(OH)D levels were excluded. Rats in both control + metformin, and, vitamin D deficient + metformin groups were treated orally (by oral gavage) with metformin (CID Co., Egypt) in a dose of 300

mg/kg/day [27] from the start of the 4th week (for the last 4 weeks of the study). Rats in vitamin D deficient + vitamin D group were treated orally with vitamin D3 (Vidrop, cholecalciferol 2800 IU/ml; MUP Co., Egypt) in a dose of 500IU/kg/day

in corn oil [28] from the start of the 4th week (for the last 4 weeks of the study). Rats of control and control + metformin groups received normal saline (1ml/rat i.p.) concomitant with paricalcitol in the other groups.

Table (1): Experimental design.

	Control	Control + metformin	Vitamin D deficient	Vitamin D deficient + metformin	Vitamin D deficient + vitamin D
Number of rats	8	8	8	8	8
Ordinary diet along the study	,	,	,	,	,
Vitamin D deficient diet along the study	,	,	,	,	,
Paricalcitol i.p. on days 1, 3, 5, 8, 10, and 12	,	,	,	,	,
Normal saline i.p. on days 1, 3, 5, 8, 10, and 12	,	,	,	,	,
Vitamin D3 orally for the last 4 weeks of the study	,	,	,	,	,
Metformin orally for the last 4 weeks of the study	,	,	,	,	,
Duration of study (weeks)	7	7	7	7	7

i.p., intraperitoneal.

Twenty-four hours after the last drug administration, all rats were weighed and were sacrificed via an overdose of general anesthetic (thiopental sodium, 50mg/kg), and the whole blood samples were withdrawn using the intracardiac method [29]. Collected blood was left at room temperature for 30 minutes to clot, then centrifuged at 3000RPM for 15 minutes and sera were separated and stored at -20°C until assay. Serum T3, T4, TSH, 25(OH)D, 1,25(OH)2D and reverse T3 (rT3) were measured using commercial kits (MyBioSource, Inc., San Diego, USA). After rats were sacrificed, the liver and thyroid gland of each rat were immediately excised. The liver was washed in buffer solution at 4°C, then gently wiped and stored at -80°C. Deiodinase 3 activity in the liver homogenate was measured with corresponding kits (MyBioSource, Inc., San Diego, USA), following the manufacturer's instructions [30]. Thyroid glands were weighed, and fixed in 10% neutral buffered formalin and embedded in paraffin. The relative weight of thyroid gland was expressed per 100g body weight [31].

Histopathological studies:

Sections of thyroid gland (5 ~~µm~~ thick) were prepared and stained with the followings:

- Hematoxylin and eosin (H & E) and Mallory's trichrome to detect collagen fibers [32].
- Ki67 immunohistochemistry: Deparaffinized sections were pretreated for antigen retrieval via scientific microwave treatment in 0.01mol/l citrate buffer (pH 6.0). After a 10-min incubation in 3% H₂O₂ in deionized water to inhibit endogenous peroxidase activity, the sections were incubated with anti-rat Ki67 monoclonal antibody (MIB-

5) diluted 1:50 in ChemMate antibody diluent. After washing with Phosphate-Buffered Saline (PBS), the sections were incubated with biotinylated anti-rabbit and anti-mouse immunoglobulins for 30min and subsequently with streptavidin-conjugated horseradish peroxidase for 30min using an LSAB-2 system-HRP. Antibody binding was visualized via incubation of the sections with 3, 3'-diaminobenzidine (DAB) using a liquid DAB + substrate chromogen system.

Light microscopic examination and photography were done for all thyroid stained sections by Leica DM500 photomicroscope (German).

Histomorphometric studies:

The morphometric study was performed using Image J software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). Follicular Epithelial Height (FEH), Follicular Area (FA) and external Follicular Diameter (FD) were measured in H & E stained thyroid sections. Histomorphometric studies were done by Abercrombie's method [33]. Area percent of collagen fibers in Mallory trichrome stained sections, and number of ki67 positive thyrocytes in the wall of the thyroid follicle in immune stained sections were measured. All measurements were performed in 6 non overlapping fields in 6 random sections in 6 different rats in each group at magnification X400 in H & E and Mallory trichrome stained and X1000 in ki67 immunostained sections.

Statistical analysis:

The results were expressed as mean ± Standard Deviation (SD). For statistical significance, one-

way ANOVA and Tukey HSD for Post hoc multiple comparisons were used to compare means. The software, IBM Statistical Package for Social Sciences (SPSS) Version 26 Software for Windows (SPSS, Inc., Chicago, IL, USA), was used for that purpose. Also, Graph Pad Prism (Version 8 Software for Windows) was used to analyze the Pearson's correlation coefficient between serum levels of 25(OH)D and some studied parameters within vitamin D deficient group. Significance was considered with p -value ≤ 0.05 .

Results

Changes in vitamin D deficient group: (Table 2):

Final body weight (249.9±7.7), final BMI (0.68±0.04), thyroid weight (14.26±0.29) and relative thyroid weight (5.71±0.25) were significantly increased ($p<0.001$) when compared with those in the control group (207.1±9.1, 0.57±0.05, 10.11±0.27 and 4.91±0.31, respectively). Serological investigation declared a significant ($p<0.001$) increase in both TSH (9.48±0.09) and rT3 (124.8±0.94) levels when compared with that in control group (7.08±0.13 and 84.89±0.29, respectively), with a significant ($p<0.001$) decline in T3 (0.95±0.06), T4 (5.07±0.1), 25(OH)D (21.63±2) and 1,25(OH)2D (39.5±2) levels in comparison to those in the control group (1.41±0.02, 6.4±0.03, 71±2.27 and 130.38±3.11, respectively). Hepatic deiodinase 3 activity (11.85±0.04) was significantly ($p<0.001$) elevated in comparison to the control group (9.08±0.06).

Effects of metformin treatment on vitamin D deficient rats (changes in vitamin D deficient + metformin group): (Table 2):

Final body weight (237.8±7.1), thyroid weight (11.1±0.23) and relative thyroid weight (4.69±0.2) were significantly reduced ($p<0.05$) when compared with those in the vitamin D deficient group (249.9±7.7, 14.26±0.29 and 5.71±0.25, respectively). Serological investigation declared only a significant ($p<0.001$) decrease in TSH (8.17±0.15) level when compared with that in vitamin D deficient group (9.48±0.09).

Effects of vitamin D treatment on vitamin D deficient rats (changes in vitamin D deficient + vitamin D group): (Table 2):

Final body weight (213.1±6.3), final BMI (0.57±0.03), thyroid weight (10.16±0.28) and relative thyroid weight (4.78±0.2) were significantly decreased ($p<0.001$) when compared with those in the vitamin D deficient group (249.9±7.7, 0.68±0.04, 14.26±0.29 and 5.71±0.25, respectively). Serological investigation declared a significant ($p<0.001$) decrease in both TSH (7.09±0.14) and rT3 (84.25±1.56) levels when compared with that in vitamin D deficient group (9.48±0.09 and 124.8±0.94, respectively), with a significant ($p<0.001$) increase in T3 (1.39±0.02), T4 (6.39±0.04), 25(OH)D (70.13±2.23) and 1,25(OH)2D (130.5±3.96) levels in comparison to those in the vitamin D deficient group (0.95±0.06, 5.07±0.1, 21.63±2 and 39.5±2, respectively). Hepatic deiodinase 3 activity (9.09±0.04) was significantly ($p<0.001$) reduced in comparison to the vitamin D deficient group (11.85±0.04).

Table (2): Biochemical changes among different groups.

	Control	Control + metformin	Vitamin D deficient	Vitamin D deficient + metformin	Vitamin D deficient + vitamin D
Final body weight (g)	207.1±9.1	202.5±8.3	249.9±7.7 ^{a&b}	237.8±7.1 ^{a,b,c}	213.1±6.3 ^{d&e}
Final BMI (g/cm ²)	0.57±0.05	0.55±0.04	0.68±0.04 ^a	0.64±0.04 ^{f,b}	0.57±0.03 ^{g,d&h}
Thyroid weight (mg)	10.11±0.27	10.19±0.2	14.26±0.29 ^{a&b}	11.1±0.23 ^{a,b,d}	10.16±0.28 ^{d&e}
Relative thyroid weight (mg/100g final body weight)	4.91±0.31	5.04±0.18	5.71±0.25 ^{a&b}	4.69±0.2 ^{i,d}	4.78±0.2 ^d
Serum TSH (ng/ml)	7.08±0.13	6.99±0.07	9.48±0.09 ^{a&b}	8.17±0.15 ^{a,b,d}	7.09±0.14 ^{d&e}
Serum T3 (ng/ml)	1.41±0.02	1.39±0.03	0.95±0.06 ^{a&b}	1±0.07 ^{a,b}	1.39±0.02 ^{d&e}
Serum T4 (ng/ml)	6.4±0.03	6.39±0.03	5.07±0.1 ^{a&b}	5.05±0.11 ^{a,b}	6.39±0.04 ^{d&e}
Serum 25(OH)D (ng/ml)	71±2.27	70±2.27	21.63±2 ^{a&b}	22.63±1.85 ^{a,b}	70.13±2.23 ^{d&e}
Serum 1,25(OH)2D (pg/ml)	130.38±3.11	129.13±3.48	39.5±2 ^{a&b}	39.5±1.6 ^{a,b}	130.5±3.96 ^{d&e}
Hepatic deiodinase 3 (D3) activity (ng/g protein)	9.08±0.06	9.07±0.09	11.85±0.04 ^{a&b}	11.84±0.07 ^{a,b}	9.09±0.04 ^{d&e}
Serum rT3 (ng/L)	84.89±0.29	84.86±0.51	124.8±0.94 ^{a&b}	124.41±1.36 ^{a,b}	84.25±1.56 ^{d&e}

Data was expressed as Mean ± SD.

a: $p<0.001$ in comparison with control group.
 b: $p<0.001$ in comparison with control + Metformin group.
 c: $p<0.05$ in comparison with vitamin D deficient group.
 d: $p<0.001$ in comparison with vitamin D deficient group.
 e: $p<0.001$ in comparison with vitamin D deficient + Metformin group.
 f: $p<0.01$ in comparison with control group.
 g: $p<0.01$ in comparison with control + Metformin group.

h: $p<0.01$ in comparison with vitamin D deficient + Metformin group.
 i: $p<0.05$ in comparison with control + Metformin group.
 BMI: Body Mass Index.
 TSH: Thyroid Stimulating Hormone.
 T3: Triiodothyronine.
 T4: Thyroxine.
 rT3: Reverse T3.

Pearson correlations within vitamin D deficient group between serum 25(OH)D and some studied parameters: Fig. (1):

A significant negative correlation was reported between serum 25(OH)D and each of BMI ($r =$

-0.981 , $p < 0.001$), serum TSH (-0.826 , $p < 0.05$), relative thyroid weight ($r = -0.973$, $p < 0.001$), hepatic deiodinase activity ($r = -0.799$, $p < 0.05$) and serum rT3 ($r = -0.809$, $p < 0.05$), but it was positively correlated to serum level of both T3 ($r = 0.881$, $p < 0.01$) and T4 ($r = 0.939$, $p < 0.001$).

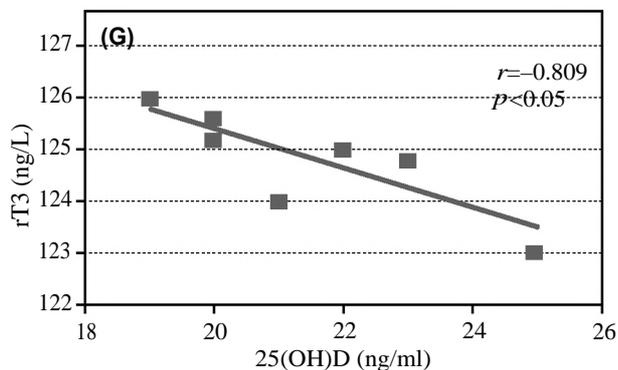
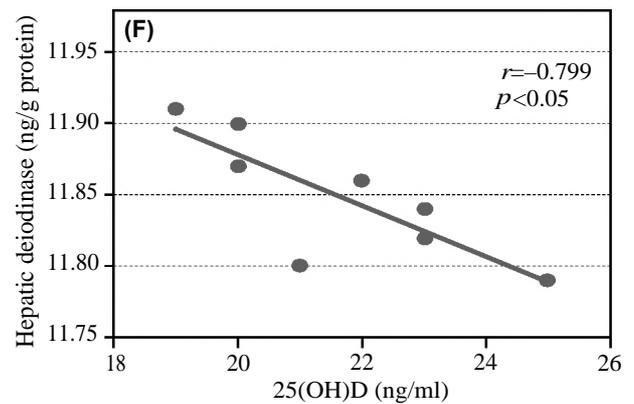
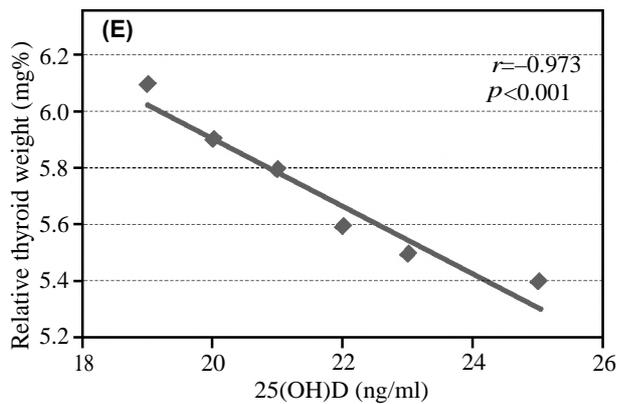
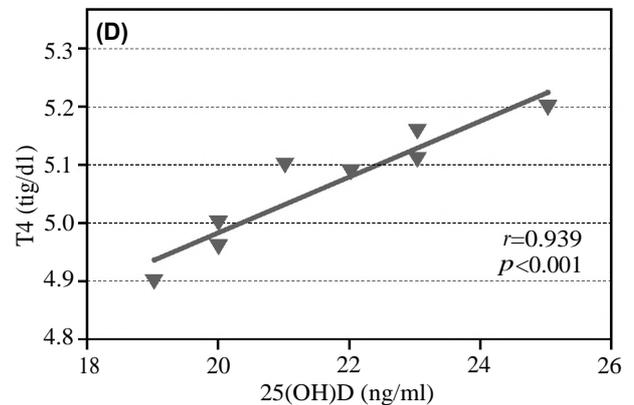
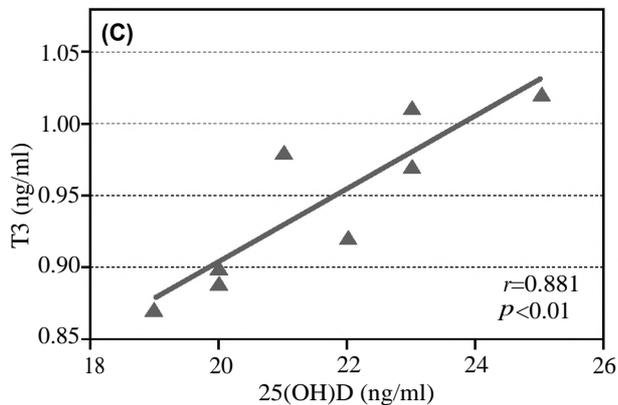
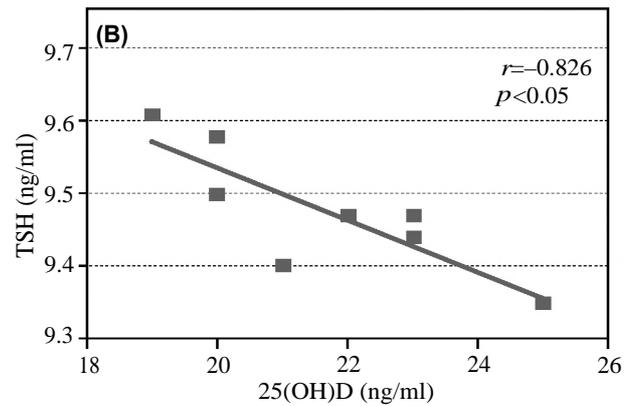
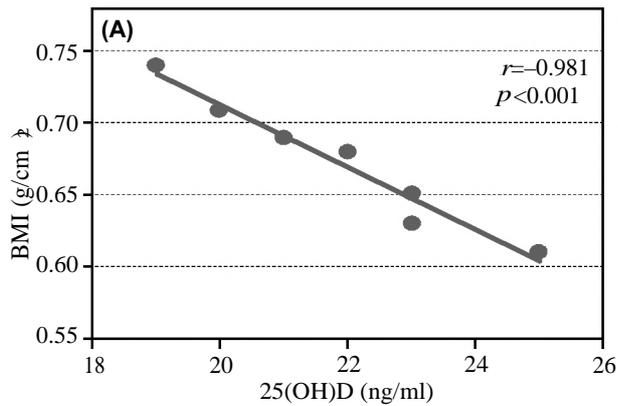


Fig. (1): Correlation coefficient (r) within the vitamin D deficient group between serum 25(OH)D and each of BMI (A), serum TSH (B), serum T3 (C), serum T4 (D), relative thyroid weight (E), hepatic deiodinase (F) and serum rT3 (G).

Light microscopic histological studies:

As comparing the histopathological and the immunohistochemical results in the control and control metformin treated groups, the results showed nearly similar findings. So, we used control group to be compared with other groups.

Hematoxylin & Eosin (H&E) staining Fig. (2A-E):

Thyroid gland of control and control metformin treated rats showed normal thyroid follicles of various sizes, lined with simple cuboidal epithelium with cells that have rounded nuclei and surrounding central lumen filled with homogenous acidophilic colloid, blood vessels, in the narrow interfollicular spaces. Fig (2A).

Thyroid gland of vitamin D deficient group showed that most of follicles are destroyed as there are many disorganized and damaged follicles with a wide interfollicular space. Thyroid follicles appeared with variable activity, where some follicles showed low cuboidal cells with apparently rounded nuclei and the thyrocytes of most of the follicles have vacuolated cytoplasm (arrow head) in the lining epithelium of the follicles and few follicles appear distended with flattening of the lining epithelium with flat nuclei. Some follicles appear with vacuolated colloid and other follicles appear involuted with minimal amount of colloid or no colloid, Fig. (2B,C). Some follicles showed shedding of epithelial lining, with desquamated follicular cells in the colloid of the follicular lumens, Fig. (2B). Some follicles in vitamin D deficiency group, showed follicular hyperplasia and hyperemia with congested interfollicular blood vessels. Fig. (2C).

Thyroid follicles in either metformin treated group or vitamin D treated groups showed marked improvement, with many recovered thyroid follicles, most of the follicles appeared similar to those of the control, while few follicles appeared with vacuolar cytoplasm or vacuolated colloid. Fig. (2D,E).

Mallory's trichrome staining Fig. (3A-D):

Thyroid gland of control and control metformin treated rats showed little collagen fibers in the connective tissue septa of thyroid stroma, Fig. (3A), while there is severe fibrosis in the interfollicular space, with many collagen fibers in the connective tissue septa in vitamin D deficient group, Fig. (3B). Vitamin D deficient rats treated with metformin showed only moderate amount of collagen fibers, Fig. (3C). Vitamin D deficient rats treated with vitamin D revealed few collagen fibers in the connective tissue septa between the follicles, almost resembling control ones, Fig. (3D).

Immunohistochemistry staining with ki67 Fig. (4A-D):

Control and control metformin treated rats showed weak immunohistochemical ki67 expression in nuclei of follicular cells., Fig. (4A), and marked strong (dark brown) immunohistochemical of ki67 expression in vitamin D deficient group, Fig. (4B). Vitamin D deficient rats treated with metformin showed reduction of immunohistochemical of ki67 expression in nuclei of follicular cells (arrows) more or less to normal level, Fig. (4C). While obvious reduction of immunohistochemical of ki67 expression is seen in vitamin D deficient rats treated with vitamin D, more or less to normal level, Fig. (4D).

Statistical analysis of histological studies:

The statistical analysis of the mean values of the numbers of positive thyrocytes in the wall of the follicle in different experimental groups showed no significant difference in the number of the immunopositive thyrocytes between the control and the control treated with metformin rats. But there was a significant increase in the number of the immunopositive thyrocytes in the vitamin D deficiency compared to the control group. In contrast, in vitamin D deficient rats treated with metformin there was a significant decrease in the number of the immunopositive thyrocytes compared to vitamin D deficiency but still a significant difference from the control group. However, treatment of vitamin D deficient rats with vitamin D revealed a significant decrease in the number of the immunopositive thyrocytes compared to vitamin D deficient rats, and vitamin D deficient rats treated with metformin and revealed a non-significant difference from the control group, Fig. (5).

The statistical analysis of the mean values of the area percent of collagen fibers in different experimental groups showed non-significant difference in the area percent of collagen fibers between the control and control treated with metformin groups. But there was a significant increase in the area percent of collagen fibers in the vitamin D deficiency group compared to the control group. In contrast, in vitamin D deficient rats treated with metformin there was a significant decrease in the area percent of collagen fiber compared to vitamin D deficiency but still revealed a significant difference from the control group. However, administration of vitamin D to vitamin D deficient rats showed a significant decrease in the area percent of collagen fiber compared to vitamin D deficiency and vitamin D deficient rats treated with metformin and revealed a non-significant difference from the control group, Fig. (6).

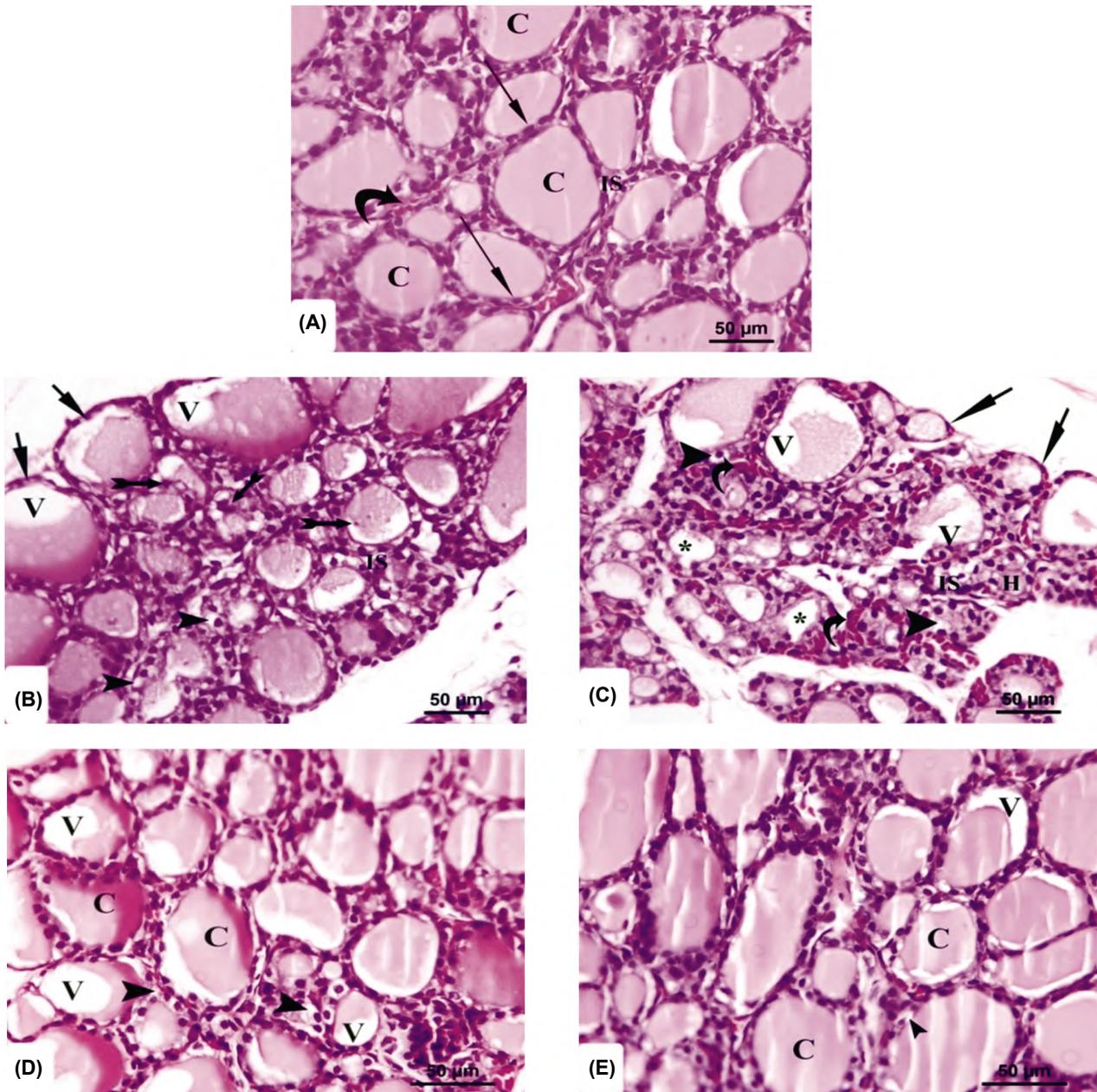


Fig. (2): (A): Photomicrograph of a section in thyroid gland of control and control metformin treated rats showing normal thyroid follicles of various sizes, lined with simple cuboidal epithelium with cells that have rounded nuclei (arrow) and surrounding central lumen filled with homogenous acidophilic colloid (C). Notice blood vessels (curved arrow), in the narrow interfollicular spaces (IS). (H & E; X400). (B): Photomicrograph of a section in thyroid gland of vitamin D deficient rats showing disorganized and damaged follicles with a wide interfollicular space (IS). Thyroid follicles appeared with variable activity, where some follicles show low cuboidal cells with apparently rounded nuclei and cytoplasmic vacuolations (arrow head) and few follicles appear distended with flattening of the lining epithelium with flat nuclei (short arrow). Some follicles appear with vacuolated colloid (V) with large interfollicular spaces (IS). Some follicles showed shedding of epithelial lining, with desquamated follicular cells in the colloid of the follicular lumens (bifid arrow). (H & E; X400). (C): Photomicrograph of a section in thyroid gland of vitamin D deficient rats showing most of follicles are destroyed as there disintegration and disorganization of thyroid follicles, few follicles appear with low cuboidal cells with apparently rounded nuclei, showing follicular hyperplasia (H) with large interfollicular spaces (IS), other follicles appear with flattening of the lining epithelium with flat nuclei (short arrow), some follicles appear with vacuolated colloid (V) and other follicles appear involuted with minimal amount of colloid or no colloid (astrix). The thyrocytes of most of the follicles have vacuolated cytoplasm (arrow head) in the lining epithelium of the follicles. Notice hyperemia with congested interfollicular blood vessels (curved arrow). (H & E; X400). (D): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with metformin showing marked improvement of the thyroid follicles (C), most thyroid follicles appeared similar to those of the control, while few follicles appeared with vacuolar cytoplasm (arrow head) or vacuolated colloid (C). (H & E; X400). (E): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with vitamin D revealing many recovered thyroid follicles (C), most thyroid follicles appeared similar to those of the control, while few follicles appeared with vacuolar cytoplasm (arrow head) or vacuolated colloid (C). (H & E; X400).

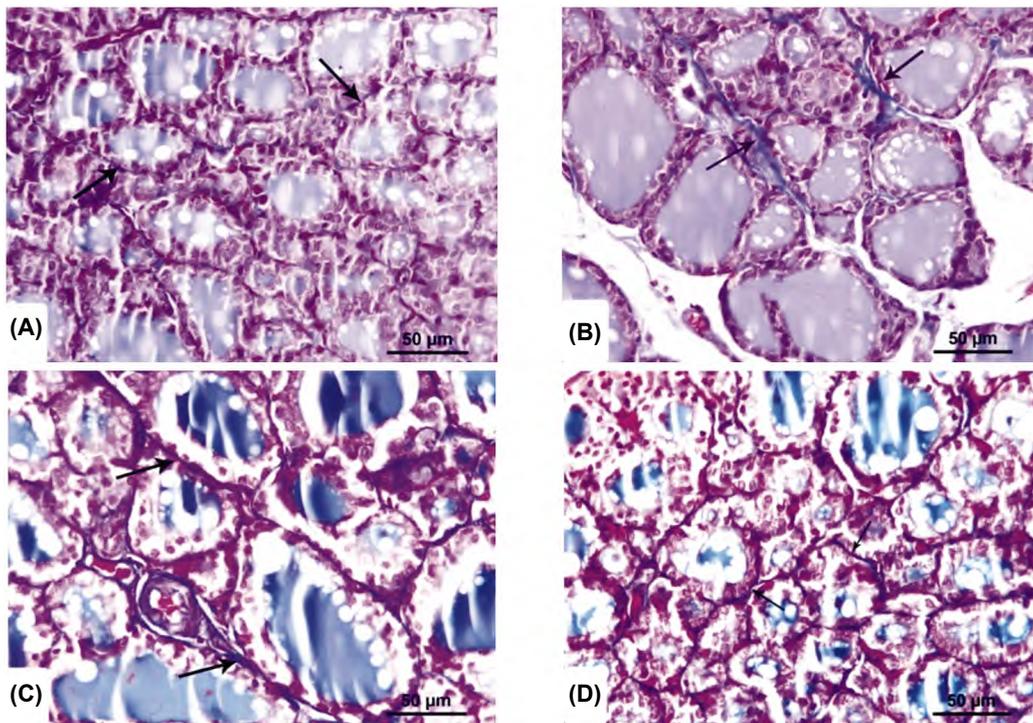


Fig. (3): (A): Photomicrograph of a section in thyroid gland of control and control metformin treated rats showing little collagen fibers in the connective tissue septa of thyroid stroma; (arrow) (Mallory's trichrome X400). (B): Photomicrograph of a section in thyroid gland of vitamin D deficient rats showing severe fibrosis in the interfollicular space, with many collagen fibers in the connective tissue septa (arrow) (Mallory's trichrome X400). (C): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with metformin showing moderate amount of collagen fibers in the connective tissue septa between the follicles (arrow) (Mallory's trichrome X400). (D): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with vitamin D revealing few collagen fibers in the connective tissue septa between the follicles (arrow) (Mallory's trichrome X400).

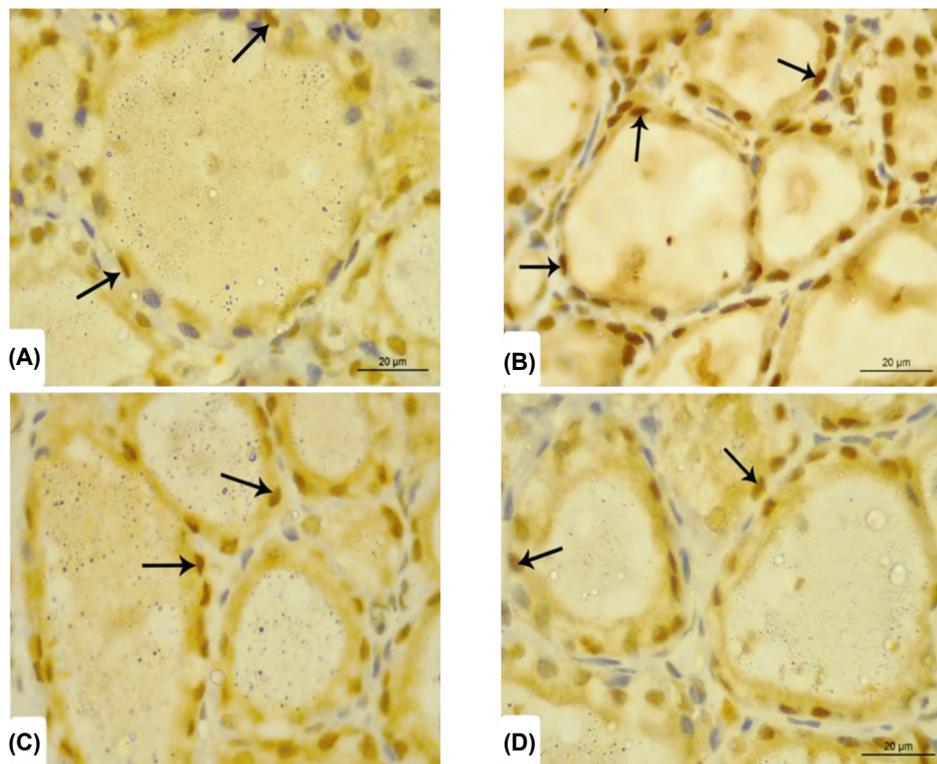


Fig. (4): (A): Photomicrograph of a section in thyroid gland of control and control with metformin treated rats showed weak immunohistochemical ki67 expression in nuclei of follicular cells (arrow). (ki67 immunohistochemical staining X1000). (B): Photomicrograph of a section in thyroid gland of vitamin D deficient rats showing marked strong immunohistochemical of ki67 expression in nuclei of follicular cells (arrows). (ki67 immunohistochemical staining X1000). (C): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with metformin showing reduction of immunohistochemical of ki67 expression in nuclei of follicular cells (arrows) more or less to normal level (ki67 immunohistochemical staining; X1000). (D): Photomicrograph of a section in thyroid gland of vitamin D deficient rats treated with vitamin D showing obvious reduction of immunohistochemical of ki67 expression in nuclei of follicular cells (arrows) more or less to normal level (ki67 immunohistochemical staining; X1000).

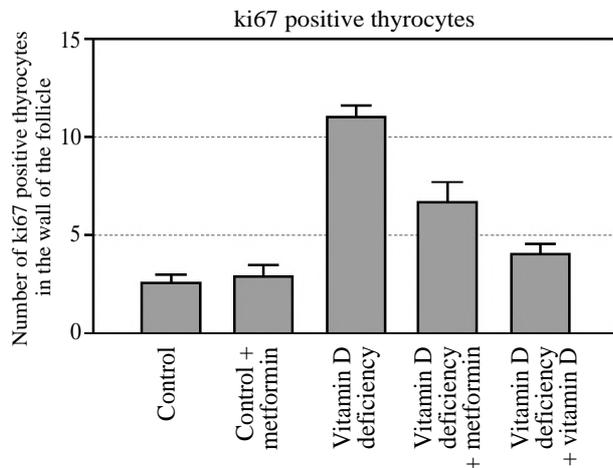


Fig. (5): Bar chart showing the statistical analysis of the mean values of the numbers of positive thyrocytes in the wall of the follicle in different experimental groups.

Values were represented as the mean ± SE.
 *: Significant difference compared to the control groups (control and control + metformin), $p < 0.05$.
 #: Significant difference compared to the vitamin D deficient group, $p < 0.05$.
 @: Significant difference compared to the vitamin D deficiency + metformin group, $p < 0.05$.

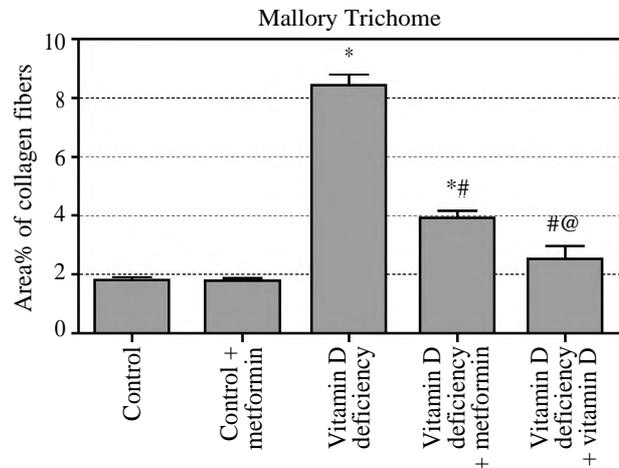


Fig. (6): Bar chart showing the statistical analysis of the mean values of the area percent of collagen fibers in different experimental groups.

Values were represented as the mean ± SE.
 *: Significant difference compared to the control groups (control and control treated with metformin), $p < 0.05$.
 #: Significant difference compared to the vitamin D deficiency group, $p < 0.05$.
 @: Significant difference compared to the vitamin D deficient rats treated with metformin, $p < 0.05$.

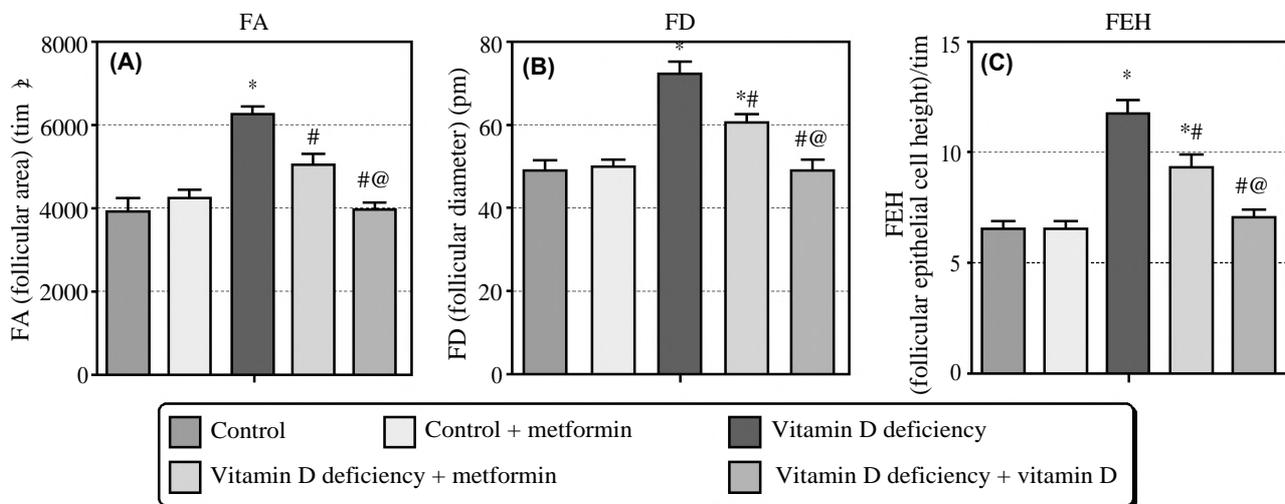


Fig. (7): Bar chart showing the statistical analysis of the mean values of the follicular area, follicular diameter and follicular epithelial height in different experimental groups.

Values were represented as the mean ± SE.
 *: Significant difference compared to the control groups (control and control treated with metformin), $p < 0.05$.
 #: Significant difference compared to the vitamin D deficiency group, $p < 0.05$.
 @: Significant difference compared to the vitamin D deficient rats treated with metformin, $p < 0.05$.

The statistical analysis of the mean values of the follicular area Fig. (7A), follicular diameter Fig. (7B) and the follicular epithelial height Fig. (7C) in different experimental groups showed non-significant difference in their values between the control and the control treated with metformin. In contrary, there was a significant increase in the follicular area, follicular diameter and the follicular epithelial height in the vitamin D deficiency compared to the control group. However, in vitamin D deficient rats treated with metformin revealed a

significant decrease in the follicular diameter and the follicular epithelial height compared to vitamin D deficiency but the follicular area revealed decrease in its value but still showed non-significant difference from the vitamin D deficiency group but all parameters still revealed significant difference from the control group.

In contrast, administration of vitamin D to vitamin D deficient rats revealed a significant decrease in the follicular area, follicular diameter

and the follicular epithelial height compared to vitamin D deficiency and non-significant difference from the control group. In addition, the follicular area, the follicular diameter and the follicular epithelial height revealed a significant difference from the vitamin D deficient rats treated with metformin, Fig. (7A,B,C).

Discussion

This study declared a significant reduction in thyroid function in vitamin D deficient rats which was associated with changes in thyroid gland structure indicating occurrence of hypothyroidism that was improved after vitamin D treatment. In addition, metformin administration improved thyroid structure and decreased the elevated TSH without affecting thyroid hormone levels in vitamin D deficient rats. In control rats treated with metformin (control + metformin group), there was insignificant changes in all measured parameters in comparison to the control group which confirmed that metformin had no effect on thyroid structure and function in normal rats which was supported by Díez and Iglesias [9], Fournier, et al. [7] and Lupoli, et al. [34] and Reddy, et al. [10] who recorded no change in TSH levels in euthyroid patients. In contrary, Hu, et al. [35] reported that metformin affected thyroid profile in normal rats.

In vitamin D deficient group there was a significant increase in final BMI, relative thyroid weight, hepatic deiodinase 3 activity, and, serum levels of both TSH and rT3, with a significant decline in serum levels of T3, T4, 25(OH)D and 1,25(OH)₂D levels when compared with those in the control group. These results confirmed occurrence of hypothyroidism with vitamin D deficiency which confirm that vitamin D deficiency could be considered as one of the causes of hypothyroidism in rats. These results were supported by Bhatnagar, et al. [36] and Idiculla, et al. [22] who recorded a significant decline in serum level of 25(OH)D in patients with hypothyroidism when compared to euthyroid controls. The change in thyroid function in vitamin D deficient group was asserted by histological examination of the thyroid follicles, which unveiled evident structural changes, reflecting augmented thyroid follicles activity in response to increased secretion of TSH to compensate the decreasing levels of T3 and T4. These changes were manifested by swollen vacuolated follicular cells, follicular hyperplasia, along with excessive vacuolation of the colloid. Congested blood vessels were also encountered. Badr El Dine, et al. [37] described similar features. These changes were further bolstered by morphometric and statistical

analyses that revealed a significant increase in the follicular diameter, follicular area and follicular epithelial height as compared with other groups which was in agreement with Dalvinder, et al. [33]. On treatment of vitamin D deficient rats with metformin (vitamin D deficient + metformin group), a significant reduction in relative thyroid weight and serum TSH was detected. This result confirmed that metformin improved thyroid structure possibly through lowering serum TSH without affecting thyroid function. This was supported by Morteza Taghavi, et al. [38], Distiller, et al. [39], Dimic, et al. [2] and Krysiak, et al. [40] who reported that metformin reduced TSH levels in both overt and subclinical hypothyroidism. Also, Blanc, et al. [41] declared that diabetic patients treated with metformin had a smaller thyroid volume and a lower risk for the formation of thyroid nodules in comparison with the metformin untreated diabetic patients. Chau-Van, et al. [42] and Labuzek, et al. [43] owed the effect of metformin to its ability to cross the blood-brain barrier and to inhibit the activity of hypothalamic Adenosine Monophosphate activated Protein Kinase (AMPK), which affected the role of T3 on the hypothalamus. On the contrary, Hu, et al. [35] recorded that rats treated with metformin showed symptoms like hyperthyroidism, such as irritability, diarrhea and weight loss, as well as a decrease in TSH and a marked increase in T3 and T4 levels. This discrepancy may be referred to different duration and conditions of the study. Treatment of vitamin D deficient rats with vitamin D (vitamin D deficient + vitamin D group) decreased final BMI, relative thyroid weight, hepatic deiodinase 3 activity, and, serum levels of both TSH and rT3, with a significant increase in serum levels of T3, T4, 25(OH)D and 1,25(OH)₂D. These results emphasized that vitamin D supplementation deteriorated thyroid structure and function changes occurred with vitamin D deficiency. This was supported by Alrefaie and Awad [24] who reported that vitamin D3 intake in diabetic rats significantly corrected the alterations in thyroid profile and they owed this effect to the role of vitamin D in increasing deiodinase 2 expression and consequently peripheral conversion of T4 into T3. Regarding results of the current study, the improvement in the level of thyroid hormones in vitamin D deficient rats treated with vitamin D could be owed to reduction in hepatic deiodinase 3 activity which reduced degradation of thyroid hormones in the liver and this was evidenced earlier by Kester, et al. [44] and Darras and Van [45] who confirmed that hepatic deiodinase 3 is the major inactivating pathway for thyroid hormones, catalyzing degradation of T3 to 3, 3-diiodothyronine

and conversion of T4 to the inactive metabolite rT3 and this could be an explanation to the decrease in rT3 serum level in vitamin D deficient + vitamin D group in this study. Talaei, et al. [46] reported that vitamin D supplementation among hypothyroid patients improved serum TSH, but it did not alter serum T3 and T4 levels. The discrepancy between the results of the previous study and the results of the current study may be due to species difference and the cause of disturbed thyroid profile in this study which was vitamin D deficiency and thus treatment of the cause corrected the disturbance. Tissue fibrosis is known to cause organ dysfunction. To determine the degree of fibrosis in the thyroid tissues of the rats, Masson's trichrome staining was carried out. The staining showed a significant more fibrosis in the vitamin D deficiency group than in the other groups. The degree of fibrosis was significantly decreased after treatment with vitamin D which was supported by Liu, et al. [47] who noticed that vitamin D3 reduced fibrosis and apoptosis in diabetic rat testis and they concluded that vitamin D3 supplementation could improve testicular function through transforming growth factor-beta 1 (TGF- β 1) signaling pathway modulation. Within vitamin D deficient group, a significant negative association between serum 25(OH)D and each of final BMI, serum TSH, relative thyroid weight, hepatic deiodinase 3 activity and serum rT3, was detected, with a positive correlation between serum level of 25(OH)D and that of both T3 and T4. These findings were in agreement with Mackawy, et al. [20] and Kim [48] who suggested that 25(OH)D serum level deficiency was significantly correlated with the degree and severity of hypothyroidism. Also, Zhang, et al. [49] found that serum TSH concentrations were negatively correlated with that of 25(OH)D. In contrary, Goswami, et al. [23] reported no relationship between serum level of 25(OH)D and thyroid function. This discrepancy may be related to species differences. Immunohistochemical analysis in this study showed that the vitamin D deficiency group had the highest proportion of Ki67-positive thyrocytes compared with the other groups which indicated increased number of proliferating cells in thyroid gland in vitamin D deficient rats. Ki67 was used as a proliferation-associated nuclear antigen that provided a powerful tool to detect the replicative cells more accurately than Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry [50,51]. In vitamin D deficient rats treated with either metformin or vitamin D, the number of Ki67-positive thyrocytes was significantly decreased, in comparison to the vitamin D deficient rats, reflected the anti-proliferative effects of both metformin or vitamin

D which was in agreement with Fleet [52], Gocek and Studzinski [53], Adams and Hewison [54], Baur, et al. [55], Ma, et al. [56] and Thakur, et al. [57]. Adams and Hewison [54] reported that vitamin D is essential in regulation of cell proliferation and differentiation. In an epidemiologic study, Thakur, et al. [57] recorded that metformin was associated with reduction in incidence of certain cancers, and improved response to cancer therapy. Limitations of this study included that it was conducted on rats and the results may be different from human. Also, effect of vitamin D supplementation on the control rats was not studied. Moreover, only hepatic deiodinase 3 activity was assessed while activities of other deiodinases were not investigated. Also, anti-inflammatory and anti-oxidant activities of vitamin D and metformin were not assessed.

Conclusion:

As vitamin D deficiency decreased thyroid function through affecting hepatic deiodinase 3 activity and this change was deteriorated with vitamin D supplementation, thus, monitoring of vitamin D levels and its supplementation may play a role in management of hypothyroidism. Also, metformin exerted an anti-proliferative activity, improved thyroid structure and lowered TSH without affecting serum levels of T4 or T3, thus it could provide a rationale for an innovative therapy of thyroid proliferative diseases.

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Conflict of interest:

Nothing.

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تغير وظائف الغدة الدرقية في نموذج لنقص فيتامين (د) في الجردان وتأثير علاج فيتامين (د) وميتفورمين

خلفية البحث: أظهرت دراسات سابقة خلافاً حول ارتباط نقص فيتامين (د) ونقص الغدة الدرقية وعلى حد علمنا لم تكن هناك دراسة أجريت لدراسة تأثير نقص فيتامين (د) على وظيفة الغدة الدرقية. كما أن الميتفورمين هو عقار يستخدم على نطاق واسع في علاج مرض السكري وأيضاً كانت هناك بيانات متناقضة حول تأثيره على وظائف الغدة الدرقية.

الهدف من الدراسة: تحديد ما إذا كان نقص فيتامين (د) يعتبر سبباً في قصور الغدة الدرقية أم لا. وأيضاً لمعرفة تأثير علاج الميتفورمين على وظيفة الغدة الدرقية في نموذج نقص فيتامين (د) في الجردان.

مواد وطرق البحث: تم تقسيم أربعين من ذكور الجردان البيضاء البالغين بشكل عشوائي إلى ٥ مجموعات متساوية كل مجموعة بها ٨ جردان كما يلي:

- المجموعة الأولى: المجموعة الضابطة.
- المجموعة الثانية: المجموعة الضابطة المعالجة بالميتفورمين.
- المجموعة الثالثة: مجموعة نقص فيتامين (د).
- المجموعة الرابعة: مجموعة نقص فيتامين (د) ومعالجة بالميتفورمين.
- المجموعة الخامسة: مجموعة نقص فيتامين (د) ومعالجة بفيتامين (د).

النتائج: زاد نموذج نقص فيتامين (د) بشكل ذات دلالة إحصائية من مؤشر كتلة الجسم، الوزن النسبي للغدة الدرقية، ومستوى هرموني TSH و rT3 بمصل الدم ونشاط الإنزيم الكبدي دي أيودينيز ٣، لكنه خفض بدرجة ذات دلالة إحصائية من مستوى كل من T4, T3 و 25(OH)D في مصل الدم بالمقارنة مع المجموعة الضابطة. كما قلل علاج الميتفورمين في الجردان التي تعاني من نقص فيتامين (د) بشكل ذات دلالة إحصائية من الوزن النسبي للغدة الدرقية مع انخفاض ذو دلالة إحصائية في مستوى هرمون TSH بالمقارنة مع مجموعة نقص فيتامين (د). كما تمت ملاحظة وجود ارتباط سلبي ذات دلالة إحصائية بين مستوى فيتامين (د) في مصل الدم وكل من مؤشر كتلة الجسم، مستوى TSH في مصل الدم، وزن الغدة الدرقية النسبي ومستوى rT3 في مصل الدم، ولكن كان مرتبطاً بشكل إيجابي بصورة ذات دلالة إحصائية بمستوى T3 و T4 بمصل الدم. كما أظهرت دراسات التشريح النسيجية في الغدة الدرقية للجردان التي تعاني من نقص فيتامين (د) زيادة كمية الغروانية ووجد أن بالخلايا أصبحت مكعبة ذات النواة قاعدية والعديد من التغيرات التي تؤكد حدوث نقص في نشاط الغدة الدرقية والتي تحسنت باستخدام كل من فيتامين (د) والميتفورمين.

الخلاصة: نقص فيتامين (د) يقلل من وظائف الغدة الدرقية من خلال تأثيره على نشاط الإنزيم الكبدي دي أيودينيز ٣ والتغير في بنية الغدة الدرقية ولقد تحسنت تلك التغيرات بالعلاج بفيتامين (د). وبالتالي، قد تلعب مراقبة مستويات فيتامين (د) والعلاج به دوراً في معالجة قصور الغدة الدرقية. كما أوضحت الدراسة أن العلاج بالميتفورمين قد أدى إلى تحسين بنية الغدة الدرقية وخفض مستوى TSH في مصل الدم دون التأثير على مستويات T4 أو T3 في الجردان التي تعاني من نقص فيتامين (د). وبالتالي يمكن استخدام الميتفورمين للمشاركة في السيطرة على التغيرات الهيكلية في الغدة الدرقية.