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The Effect of Simvastatin on Thyroid Function in Experimental Autoimmune Thyroiditis Induced in Rats

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Abstract

Autoimmune thyroiditis or Hashimoto's thyroiditis: is the most common autoimmune thyroid disease. is characterized by marked lymphocytic infiltration and the presence of serum autoantibodies against Thyroid peroxidase (TPO) and Thyroglobulin (TG). Genetic predisposition and cellular and humoral immunity play a role in thyroid autoimmune pathogenesis. Antithyroid antibodies are produced by interaction between Antigen presenting cells, T lymphocytes, and B lymphocytes to fix complement. Thyroid tissue is more severely damaged by complement-dependent antibody-mediated cytotoxicity. Statins, the powerful inhibitors of HMG-CoA reductase have pleotropic effects including anti-inflammatory and immunomodulatory effects. In this current study, Experimental autoimmune thyroiditis was induced in twenty-four female Wistar rat. Simvastatin was given at a dose of 4mg/kg for 30 days. Thyroid autoantibodies and Thyroid hormones were measured by ELISA technique Results showed that Simvastatin reduced autoantibodies against TPO and TG. It also lowered serum level of TSH and increased serum level of T4. Due to the undesirable side effects associated with the current treatment options used in Autoimmune thyroiditis. Simvastatin might be a promising approach in the treatment of this disease.

Keywords: Autoimmune thyroiditis, Hashimoto's Thyroiditis, Statins, Pleotropic effects, Simvastatin.

تأثير عقار سيمفاستاتين على وظيفة الغدة الدرقية في التهاب الغدة الدرقية المناعي الذاتي التجريبي المستحث في الجرذان

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الخلاصة

التهاب الغدة الدرقية المناعي الذاتي أو التهاب الغدة الدرقية هاشيموتو، هو الأكثر شيوعا بين الامراض المناعية التي تصيب الغدة الدرقية يتميز بتسلل الخلايا الليمفاوية بشكل ملحوظ ووجود الأجسام المضادة الذاتية في الدم ضد الثايرغلوبيولين والثايروبيروكسايدسز في الغدة الدرقية. يلعب الاستعداد الوراثي والمناعة الخلوية والخلطية دورًا في التسبب في أمراض المناعة الذاتية للغدة الدرقية.

يتم إنتاج الأجسام المضادة للغدة الدرقية عن طريق التفاعل بين الخلايا المقدمة للمستضد والخلايا التائية والخلايا البائية للتثبيت المتممة. تتضرر أنسجة الغدة الدرقية بشدة بسبب السمية الخلوية المعتمدة على الأجسام المضادة بوساطة الخلية. ادوية الستاتين، وهي مثبطات قوية لانزيم "مختزل ٣-هيدروكسي-٣-ميثل جلوتاريل تميم الانزيم أ" لها تأثيرات متعددة الاتجاهات بما في ذلك التأثيرات المضادة للالتهابات والتأثيرات المناعية.

في هذه الدراسة الحالية، تم إحداث التهاب الغدة الدرقية المناعي الذاتي التجريبي في أربعة وعشرون أنثى من جرذان ويستار. تم إعطاء عقار سمفاستاتين بجرعة ٤ مجم / كجم لمدة ٣٠ يومًا. تم قياس الأجسام المضادة للغدة الدرقية وهرمونات الغدة الدرقية بواسطة تقنية الاليزا وأظهرت النتائج أن السمفاستاتين يقلل من الأجسام المضادة الذاتية ضد الثاروغلوبيولين والثايروبيروكسايديز للغدة الدرقية. كما أدى إلى خفض مستوى هرمون منبه الدرقية في الدم وزيادة مستوى هرمون الثايروكسين في المصل. بسبب الآثار الجانبية غير المرغوب فيها المرتبطة بخيارات العلاج الحالية المستخدمة في التهاب الغدة الدرقية المناعي الذاتي. قد يكون سمفاستاتين نهجًا واعدًا في علاج هذا المرض.

الكلمات المفتاحية: التهاب الغدة الدرقية المناعي الذاتي، هاشيموتو، تأثير متعدد الاتجاه، الستاتينات، سمفاستاتين

Introduction

Hashimoto's thyroiditis (HT): also called chronic lymphocytic or autoimmune thyroiditis, is the most common autoimmune thyroid disease [(1)]. The prevalence of HT is reported to be 0.3–1.5 cases per 1000 population, with a 7–10:1 female to male ratio. Between 1% and 2% of children are thought to be affected [(2)].Triiodothyronine (T3) and thyroxine (T4), which are secreted by the thyroid gland, are key players in the body's metabolism, growth, and development. Pituitary gland hormone releases thyroid stimulating hormone (TSH) that causes the release of these hormones [(3)]. Iodide is oxidized and added to amino acids in the Thyroglobulin (TG), a glycoprotein made in the thyroid cells, as part of the multi-step process that creates thyroid hormones. Thyroid peroxidase (TPO), an enzyme located in the apical membrane of thyroid cells, mediates the oxidation and integration of iodide with thyroglobulin [3]. This disease is characterized by marked lymphocytic infiltration and the presence of serum autoantibodies against TPO and TG [(4)]. Although the precise mechanism of the immune system's attack on thyroid tissue is unknown, numerous epidemiological studies have shown that the interaction of susceptible genes, the environment, and other variable have a role in the

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development of autoimmune thyroiditis [5]. Loss of tolerance to self-antigens and the onset of the auto-immune process appear to be strongly influenced by genetic predisposition. Both cellular and humoral immunity play a role in thyroid autoimmune pathogenesis [6]. Thyroid specific antigens specifically TG and TPO act as a source of self-antigenic peptides. At the outset of the illness, the thyroid develops a cluster of antigen-presenting cells (APCs), primarily dendritic cells. These cells subject naive T lymphocytes to these thyroid-specific autoantigens, resulting in the activation and clonal expansion of the latter. As a result, after this stage of the disease, autoreactive T and B cells mature in the draining lymph nodes, followed by a phase of clonal expansion [7]. APCs move from the thyroid to the lymph node that drains while consuming relevant autoantigens. Antigen-producing B lymphocytes, cytotoxic T cells, and macrophages penetrate and assemble in the thyroid gland through the proliferation of lymphocyte clones and lymphoid tissue within the thyroid gland [8]. Eventually, there is a substantial loss of thyrocytes (Cell mediated immune destruction). Through antibody-dependent, cytokine-mediated, and apoptotic routes of cytotoxity, this loss is brought on by the production of autoreactive T cells, B cells, and antibodies, which causes hypothyroidism and Hashimoto's disease [9].

Statins, are a class of medications used to treat hyperlipidemia. they are powerful inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [10]. Inhibition of this essential enzyme may have pleiotropic effects since Mevalonate, a byproduct of the HMG CoA reductase process, serves as a precursor for several other nonsteroidal isoprenoids in addition to cholesterol [11]. These pleiotropic benefits consist of atherosclerotic plaque stabilization, immunomodulation, anti-inflammatory capabilities, improved endothelial function, antioxidant activity, and anti-thrombotic action [12]. Figure 1. There is a fair amount of evidence that statins can alter immunological reactions. These include impacts on a variety of immune cells' intimal recruitment, differentiation, proliferation, and secretory activities, primarily T cells and monocyte/macrophages [13]. Glucocorticoids (GCs) are the go-to therapy for reducing inflammation and immunological activation in a number of inflammatory and autoimmune illnesses. Patients with HT receive a course of glucocorticoids to reverse the condition, preventing the onset of chronic hypothyroidism and the subsequent requirement for thyroxine replacement therapy [14]. GCs may block the release of inflammatory mediators (such cytokines and prostaglandins), reduce macrophage aggregation, interfere with T and B cell function, reduce immune cell activity, and stop antibodies from binding to receptors. GCs have particular immunomodulatory effects on T lymphocytes. Through encouraging T cells to return to the bone marrow and secondary lymphoid organs, it can lower the number of circulating T cells [15]. However, GCs have many serious side effects [12].

The aim of this study is to investigate the potential effect of simvastatin on thyroid function in experimental autoimmune thyroiditis induced in female Wistar rats.

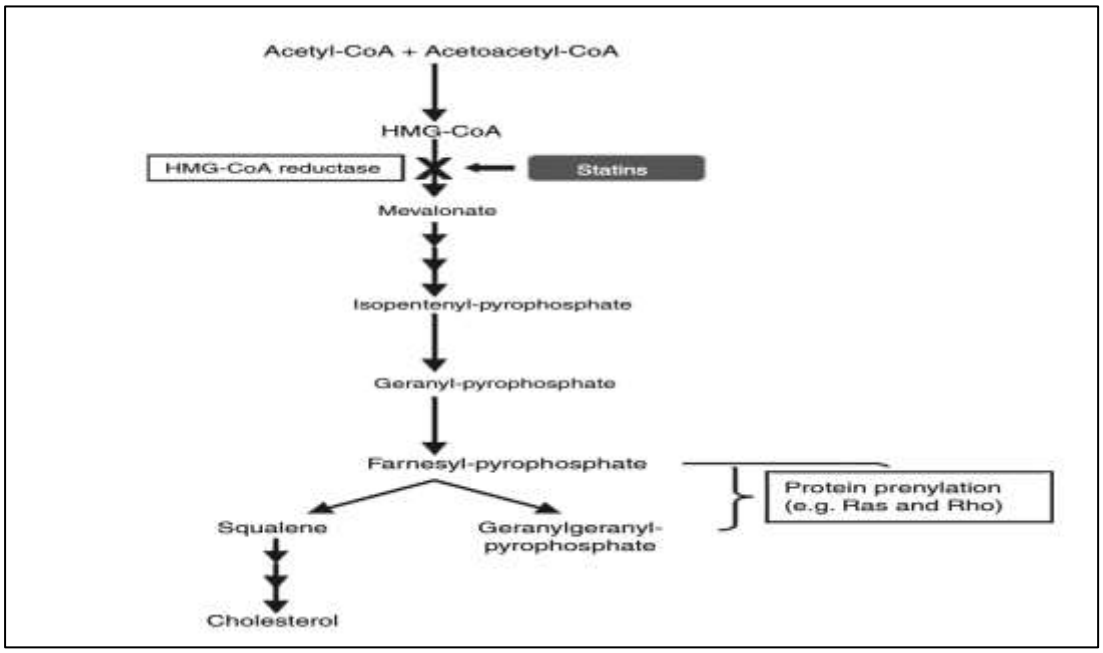

Figure 1. Mechanism of action of statins involving the mevalonate pathway. Statins prevent the production non-sterol isoprenoid byproducts which play a crucial role in the post-translational modification of numerous proteins involved in intracellular signal transduction pathways.

Materials and methods

Chemicals and drugs

Freund's Complete adjuvant (CFA) (Santa Cruz biotech. Inc. USA), Freund's incomplete adjuvant (IFA) (Sigma-Aldrich.USA), Porcine thyroglobulin (pTg) (Sigma-Aldrich.USA), Simvastatin (raw material) (Baoji Guokang Biotech. China), Prednisolone (raw material) (Baoji Guokang Biotech. China).

Reagents

Standard assay rat's kit for TSH, T4, T3 (MyBiosource, USA), Rat anti-thyroglobulin antibody (ATGA) and Rat anti-thyroid-peroxidase antibody (TPO-Ab) (MyBiosource, USA).

Experimental design

Preliminary study

A. Effective porcine thyroglobulin dose selection for the induction of autoimmune thyroiditis

Four rats were used for this study, each rat received a different volume of porcine thyroglobulin emulsion as seen in table 1**.** The emulsion was made in phosphate buffer saline (PBS) at a concentration of 2 mg/ml of porcine thyroglobulin, and it was then vortexed for 10 minutes with an equal volume of Freund's adjuvant (1:1). On various locations on the backs of the hind legs, subcutaneous injections were given to all rats. On days 0, 7, 14, and 21, injections were administered weekly. For the first immunization (day 0), complete Freund's adjuvant (CFA) was used while for the booster doses (on day 7, 14, 21) incomplete Freund's adjuvant (IFA) was used instead according to a study by Li Y. *et al*. (2021) and Song X. *et al*. (2011) [17,18]. On day 28, rats were slaughtered, and serum antithyroglobulin antibodies were measured using ELISA technique. Results showed that female rats given injections of 4 mg/ml of Porcine TG had the highest serum levels of ATGA (0.4 ml of the emulsion).

Table 1. results of preliminary study for effective porcine thyroglobulin selection.

Groups	Number of Animals	Dose of Porcine TG	ATGA level ng/ml
Group 1	1	1ml of PBS only	0.129
Group 2	1	1mg/ml (0.1 ml of emulsion)	0.144
Group 3	1	2mg\ml (0.2 ml of emulsion)	0.205
Group 4	1	4mg\ml (0.4 ml of emulsion)	0.369

B. Effective simvastatin dose selection

Twelve female Wistar rats were divided into four groups. Each group contained three female rats. Group 1 was the induction group. Treatment groups (2,3 and 4) were given different doses of simvastatin as seen in table 2. Simvastatin was given orally to rats on a daily basis for 30 days. Afterwards, rats were sacrificed and blood samples were drawn to prepare serum for ELISA to measure the level of ATGA. Group 3 showed the best results in lowering ATGA serum level with low mortality rate. Two rats of group 4 died and therefore results of this group were omitted.

Table 2. Preliminary study for selecting the effective simvastatin dose

Groups	Number of rats	Simvastatin dose
Group 1 (HT induction)	3	0.4 ml of porcine thyroglobulin emulsion
Group 2	3	2mg\kg
Group 3	3	4mg\kg
Group 4	3	8mg\kg

Table 3 . Preliminary study ELISA results for effective simvastatin dose selection.

Group	Simvastatin dose (mg/kg)	Log dose (mg/kg)	Response (ATGA ng/ml)	Response % (ATGA ng/ml)
Group 1 (HT induction)	0	0	0.30	33.67
Group 2	2	0.30	0.25	40
Group 3	4	0.60	0.11	89.38
Group 4	8	0.90	0.10	100

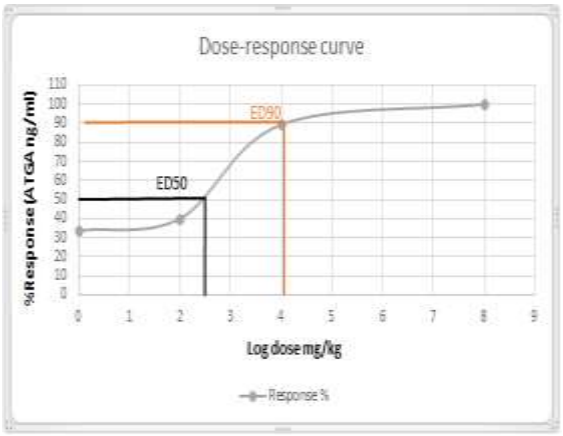

Figure 2 Dose-response curve for effective simvastatin dose selected in this study.

Experimental animals

Since the incidence of autoimmune thyroiditis is usually more prevalent in females [(2)], forty female Wistar rats weighted (200-250) g, aged (12-16) week, were used to perform this study. Rats were kept in appropriate cages with free access to water and food at the animal house of Mustansiriyiah university, College of Pharmacy. They were allowed to acclimate for fourteen days in a 12hr light/dark cycle.

The main study

Twenty-four rats were used for the main study. Experimental autoimmune thyroiditis was induced by subcutaneous injection of 0.4ml of Porcine thyroglobulin emulsion following the same procedure mentioned in the preliminary study. Rats were divided in to four groups with 6 rats in each group as follows: Group 1: normal group, rats weekly received an injection of 0.4 ml phosphate buffer saline (PBS) at various sites on the inside of the hind legs for 4 weeks.

Group 2 induction group, received 0.4 ml of porcine thyroglobulin emulsion subcutaneously weekly to induce HT. Group 3 Treatment group of oral simvastatin suspension (in distilled water and 0.5% carboxymethyl cellulose) at a dose of 4mg/kg on a daily basis for 30 days [(19)]. Simvastatin dose was choses according to preliminary study results. Table 2

Group 4 positive control group of oral prednisolone suspension (in distilled water and 0.5% carboxymethyl cellulose) at a dose of 2 mg/kg on a daily basis for 30 days [(20)].

Assessment of ELISA biomarkers

At the end of the experiment rats were given Intra-peritoneal injection of 100mg\kg of ketamine and 10mg\kg of Xylazine to render them completely anaesthetized. About 5 ml of blood was taken from the heart, let to stand at room temperature for 20 minutes, then put in tubes that enhance clotting before being centrifuged at a speed of 3000x for 15 minutes to obtain serum for ELISA technique to measure thyroid autoantibodies level (Rat anti-thyroglobulin antibody, Xinqidi biotech Co. Ltd) and (Rat anti-thyroid-peroxidase antibody, MyBiosource). Thyroid hormones are measured by ELISA technique too. (Rat TSH, T3 and T4, MyBiosource).

Statistical analysis:

Using the one-way analysis of variance (ANOVA) test in SPSS (Version 25), all data analysis (reported as mean ±standard deviation) was carried out. The statistical significance was taken into account when the P-value was less than 0.05.

Results and discussion

The effect of Simvastatin on ATGA and TPO-Ab serum level:

Table 4 displays the effect of simvastatin on serum ATGA and TPO-Ab levels in experimental autoimmune thyroiditis (EAT) induced by porcine thyroglobulin in female rats.

Table 4. Serum Anti-thyroglobulin antibody (ATGA) and thyroid peroxidase antibody (TPO-Ab) changes in Experimentally induced autoimmune thyroiditis.

Group (N=4)	Type of treatment	ATGA (ng/ml)	TPO-Ab (IU/ml)
Group 1	Phosphate buffer saline only	0.17±0.02 [b]	0.13±0.03 [b]
Group 2	Porcine thyroglobulin emulsion	0.35±0.10 [a]	0.30±0.15 [a]
Group 3	Simvastatin	0.12±0.02 [c]	0.15±0.07 [b]
Group 4	Prednisolone	0.14±0.02 [c]	0.13±0.06 [b]

The data is expressed as mean ± SD. Results with small, non-identical letters (a, b, and c) differ significantly ($p < 0.05$). Group 1, positive control, group 2, Induction, group 3&4, treatment.

Mean serum ATGA level was significantly higher ($p < 0.05$) in group 2 (the induction group) compared to Group 1. When rats were treated with Simvastatin (4mg\kg) in Group 3, mean serum ATGA level was significantly decreased ($p < 0.05$) when compared to Group 2. Group 4 also showed a significant decline in serum level of ATGA as compared to group 2. Figure 3.

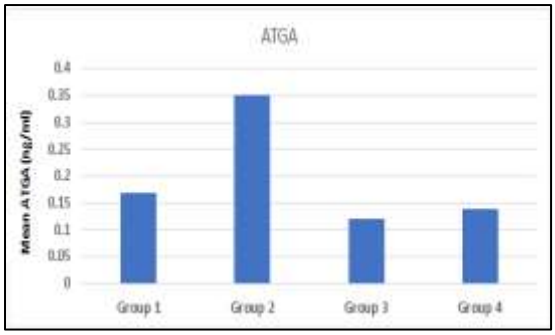

Figure 3. Serum Anti-thyroglobulin antibody (ATGA) changes in Experimentally induced autoimmune thyroiditis.

Mean serum TPO-Ab level was significantly higher ($p < 0.05$) in group 2 (the induction group) compared to Group 1. When rats were treated with Simvastatin (4mg\kg) in Group 3, mean serum TPO-Ab level was significantly decreased ($p < 0.05$) as compared to Group 2. Similarly, serum level of TPO-Ab was significantly reduced in group 4 as compared to group 2. Figure 4

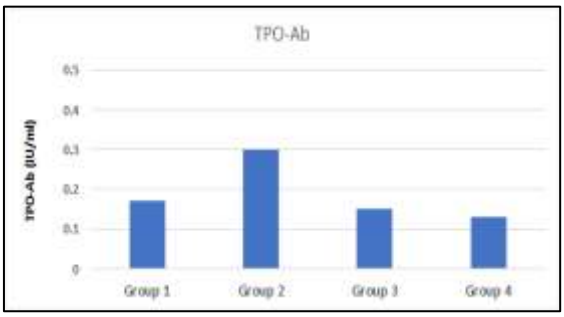

Figure 4. Serum thyroid peroxidase antibody (TPO-Ab) changes in experimentally induced autoimmune thyroiditis.

The effect of Simvastatin on thyroid function

Table 5 displays the effect of simvastatin on serum level of thyroid hormones in EAT induced by porcine thyroglobulin in female rats

Table 5. Serum level of TSH, T4 and T3 changes in Experimentally induced autoimmune thyroiditis.

Group (n=4)	Type of treatment	TSH (µIU/ml)	T4 (nmol/l)	T3 (ng/ml)
Group 1	Phosphate buffer saline only	0.43±0.07 [b]	0.50±0.08 [a]	0.52±0.05 a
Group 2	Porcine thyroglobulin emulsion	0.92±0.10 [a]	0.30±0.07 [c]	0.24±0.03 [b]
Group 3	Simvastatin	0.42±0.02 [b]	0.40±0.08 [b]	0.25±0.08 [b]
Group 4	Prednisolone	0.45±0.03 [b]	0.42±0.08 [b]	0.25±0.06 [b]

The data is expressed as mean ± SD. Results with small, non-identical letters (a, b, and c) differ significantly ($p < 0.05$). Group 1, positive control, group 2, Induction, group 3&4, treatment.

Mean serum TSH level was significantly higher ($p < 0.05$) in group 2 (the induction group) compared to Group 1. When rats were treated with Simvastatin (4mg\kg) in Group 3, mean serum TSH level was significantly decreased ($p < 0.05$) when compared to group 2. There is non-significant ($p > 0.05$) difference between group 3, 4 and 1. Figure 5

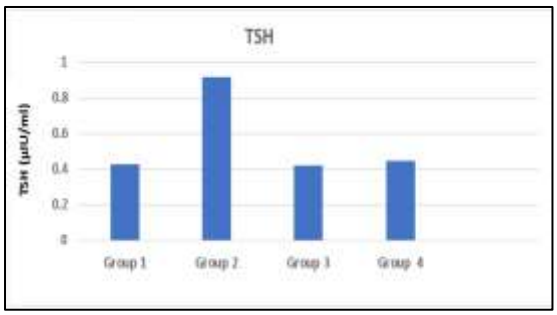

Figure 5. Serum TSH level changes in experimentally induced autoimmune thyroiditis in female rats.

While mean serum T4 level was significantly lower ($p < 0.05$) in group 2 (the induction group) compared to Group 1. When rats were treated with Simvastatin (4mg\kg) in Group 3, mean serum T4 level was significantly increased ($p < 0.05$) when compared to Group 2. There is non-significant difference between group 3 and 4. Figure 6

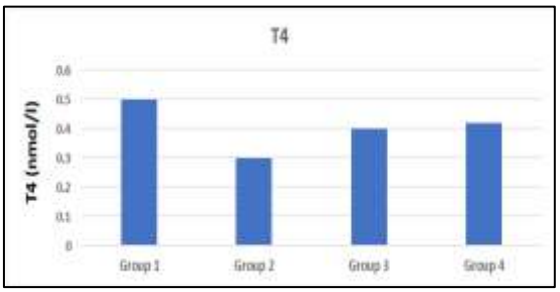

Figure 6. Serum T4 level changes in experimenttally induced autoimmune thyroiditis in female rats.

As for T3 level, it was significantly low ($p < 0.05$) in group 2 (the induction group) compared to Group 1. When rats were treated with Simvastatin (4mg\kg) in Group 3, there was no significant increase in level when compared to Group 2. Additionally, there is insignificant difference between group 3 and 4. Figure 7

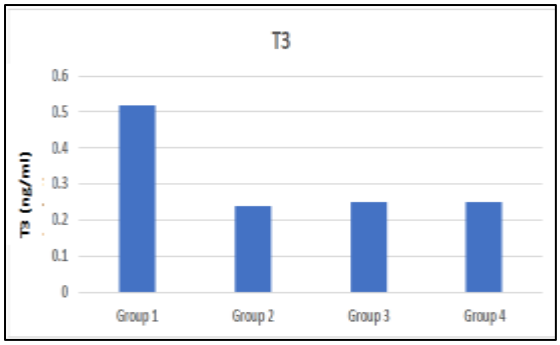

Figure 7 Serum T3 level changes in experimentally induced autoimmune thyroiditis in female rats.

In this study, rats that had been immunized with porcine thyroglobulin were utilized as an experimental model to examine the immunomodulatory effects of simvastatin in autoimmune thyroiditis. When this disease was induced, there was a significant rise in both ATGA and TPO-Ab in the serum of rats as compared with normal control group and this result is coinciding with a study done in 2018 on seventy male rats. The study showed that induction of experimental autoimmune thyroiditis by immunization of rats with porcine thyroglobulin led to a significant increase in serum levels of ATGA and TPO-Ab and was Significantly reduced by edaravone [21,22].

Results showed that Simvastatin significantly lowers the level of thyroid autoantibodies and thereby leading to an improvement of thyroid function. These results indicate that statins can modulate the immune reaction in autoimmune thyroiditis. Many mechanisms are proposed to explain their action. Statins can affect cellular trafficking, apoptosis, antigen-presenting cell (APC) maturation, and the differentiation, activation, and polarization of particular subsets of T lymphocytes [13]. The function of T cells and antigen-presenting cells (APCs) can be altered, which reduces the generation of proinflammatory mediators. Statins can directly inhibit secretion of inflammatory cytokine IL-17 by Th17 cells. They reduce the expression of the MHC (major histocompatibility complex) class II and prevent APC from mediating T helper 1 lymphocytes differentiation 13. Simvastatin has been shown to decrease the expression of IL-17A and Retinoic Acid-Related Orphan Receptors (RORs), the transcription factor that controls IL-17A synthesis in human CD4+ T cells [23].

In terms of thyroid hormones, when EAT was induced in group 2 rats, the level of blood TSH was higher than it had been in group 1 rats. Yang *et al*. (2021) reported that serum level of TSH in adult female rats was elevated following induction of EAT by immunization with Porcine TG Serum and decreased by selenium supplementation, which is consistent with this result [24].

According to study findings, treating rats with Simvastatin 4 mg/kg caused a considerable decrease in TSH levels, which is comparable to treating rats with Prednisolone.

Regarding T4 levels, study finding revealed that both serum T4 levels in group 2 rats were significantly lower than those in group 1. Simvastatin treatment resulted in a significant increase in T4 levels in the serum, which is comparable to the effects of conventional therapy in group 4.

Gullu S. *et al*. (2005) came to the conclusion that an eight-week Simvastatin treatment on HT patients changed the distribution of the lymphocyte subpopulations. These modifications were also associated with an improvement in thyroid function [25] .

According to Recent study by Wang *et al*. (2021) that Changes in total cholesterol serve as a mediating factor in the relationship between TSH levels and statin use. Statin use is associated with improvements in thyroid function. The underlying mechanism is not yet fully understood. excessive cellular cholesterol can cause ER stress, which is linked to the development of hypothyroidism. The expression of key genes involved in the manufacture of thyroid hormone by thyrocytes may be suppressed by ER stress. It has been found that the critical cholesterol receptors Niemann-Pick C1-like1 (NPC1L1) and LDL receptor are present in the thyroid glands of rats, and a high-cholesterol diet may affect thyroid function by causing ER stress. ER stress is thus one potential link between cholesterol and thyroid function. Mitochondrial oxidative stress is one of the extra possible causes of this degenerative process, among other things [(26)].

The level of T3, however, did not significantly increase. Several factors could have interfered with the conversion of T4 to T3. According to previous studies, nutritional deficits such as those in the minerals zinc, selenium, and iodine may interfere with the conversion of T4 to T3, resulting in low T3 levels. In a recent study by Kobayashi *et al*. (2021), thyroid function in those with low selenium levels was examined. While free T3 (FT3) levels were decreased, thyroid stimulating hormone (TSH) and free T4 (FT4) levels were both increased. The FT4/FT3 ratio was significantly higher in selenium deficit patients than in the control group. FT3 levels increased and the TSH, FT4, and FT4/FT3 ratio dramatically decreased when selenium was administered [27].

Conclusion

Simvastatin has an immunomodulatory effect. This effect is beneficial in suppressing autoimmune response in experimental autoimmune thyroiditis. simvastatin can alter autoimmunity by reducing antithyroglobulin and antithyroid peroxidase antibodies and therefore this will reduce thyrocytes destruction and improve thyroid

function. However, further studies are required for a longer period of time and with larger animal sample.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Funding

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Ethics Statements

This investigation was conducted in compliance with the guidelines provided by the Animal Ethics Committee of Mustansiriyah University College of Pharmacy.

Author Contribution

All authors were involved in designing the research. Yaqeen T. Mohammed conducted the experiments, collected samples and authored the manuscript. Inaam S. Arif and Nadia H. Mohammed assisted in analyzing results, reviewed the manuscript and also provided supervision throughout the project. All authors approved the final version of the manuscript.

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