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Effect of mevalonate biosynthesis inhibitor on some biochemical parameters during pregnancy in rats

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Abstract

To evaluate the detriments accompanying the inhibition of *de novo* cholesterol biosynthesis pathway using 3- hydroxy 3-methyl glutaryl coenzyme A reductase inhibitors in addition to appraising the effect of progesterone restriction on normal pregnant rats. Thirty of 1st day gestation rat females were submitted to the experiment, divided into two groups (15 rats each), control pregnant treated with deionized water and the other group treated with simvastatin (100 mg. kg^{-1} BW. day^{-1}) for twenty days by oral intubation. Serum and tissue samples from liver, ovary and placenta were obtained at the days, 1st, 15th and 20th of the treatment. Results of statistic indicated a significant ($P \leq 0.01$) decrease in the percentage of serum total cholesterol (58%), triglycerides (58%) and progesterone (60%) at the day 20th of simvastatin treatment versus to control, at the same time glucose level and alkaline phosphatase activity elevation were obtained in simvastatin- treated group. Non of albumin, total protein levels and transaminase enzymes revealed significant alteration at the end of treatment. Total cholesterol of liver, ovary and placenta manifested a significant decrease ($P \leq 0.01$) (71%, 39%, 62% respectively) since 15th day of treatment. In conclusion, the inhibition of cholesterol biosynthesis pathway during pregnancy suppresses the progesterone secretion and alter some biochemical parameters with depletion of cholesterol pool from tissues which may lead to an intense changes responsible for pregnancy failure.

***Keywords*:** Cholesterol, Progesterone, Simvastatin, Rat.
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تأثير مثبط التخليق الحيوي للميفالونات في بعض المعايير الكيميائية الحياتية أثناء فترة الحمل في الجرذان

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الخلاصة

صممت الدراسة الحالية لتقييم الآثار السلبية الناتجة عن تثبيط المسار الداخلي للتخليق الحيوي للكوليستيرول عن طريق تثبيط فعالية إنزيم 3- hydroxy 3- methyl glutaryl coenzyme A reductase فضلا عن التحري عن تأثير الحد من إفراز هورمون البروجيستيرون في إناث الجرذان الحوامل السليمة. استخدمت ثلاثون من إناث الجرذان في اليوم الأول من الحمل اذ قسمت إلى مجموعتين (١٥ حيوان/ مجموعة) عدت المجموعة الأولى مجموعة سيطرة تمت معاملتها بالماء الخالي من الأيونات بينما عوملت المجموعة الثانية بالسمفاستاتين بجرعة ١٠٠ ملغم. كغم$^{-1}$ من وزن الجسم يوميا لمدة عشرين يوما عن طريق التجريع الفموي، تم الحصول على عينات من مصل الدم وأنسجة تم أخذها من الكبد، المبيض والمشيمة في الأيام الأول، الخامس عشر والعشرين على التوالي من زمن بدء المعاملة. أشارت نتائج التحليل الإحصائي للبيانات إلى انخفاض معنوي ($P \leq 0.01$) في نسب قيم الكوليستيرول الكلي (٥٨٪)، الكليسيريدات الثلاثية (٥٨٪) والبروجستيرون (٦٠٪) في اليوم العشرين من المعاملة بالسمفاستاتين مقارنة مع مجموعة السيطرة، كما لوحظ ارتفاع في مستوى كلوكوز مصل الدم وفعالية إنزيم الفوسفاتاز القاعدي في ذات المجموعة بينما لم تختلف قيم البروتين الكلي،

ألبومين مصل الدم وفعالية الأنزيمات الناقلة للأمين معنويا لدى مقارنة قيمها بنظيراتها في مجموعة السيطرة، لوحظ من الدراسة أيضا انخفاض معنوي في مستويات الكوليستيرول الكلي لأنسجة الكبد، المبيض والمشيمة وبنسب مئوية بلغت ٧١٪، ٣٩٪ و ٦٢٪ على التوالي في اليوم العشرين مقارنة مع مجموعة السيطرة. يستنتج من هذه الدراسة أن عملية تثبيط مسار التخليق الحيوي للكوليستيرول أثناء فترة الحمل من شانها التداخل سلبا مع عملية إفراز البروجيستيرون فضلا عن دورها في اضطراب بعض المعايير الكيميائية الحياتية إضافة لكونها السبب المحوري في انخفاض مستوى الكوليستيرول داخل الأنسجة مما قد ينجم عنه بعض الخطورة والتي تهدد استمرار حالة الحمل.

Introduction

Cholesterol biosynthesis is well recognized pathway currently performed in the cytoplasm and endoplasmic reticulum of few organs including gonads from acetyl Co-A and acetoacetyl Co-A. These compounds are transformed to 3- hydroxy 3- methyl glutaryl coenzyme A HMG Co-A which reduced to mevalonate in an irreversible committed step by the action of an integral membrane enzyme in the endoplasmic reticulum, named, 3- hydroxy 3- methyl glutaryl coenzyme A reductase Using NADPH as a hydrogen source (1), cholesterol represents the precursor for steroid hormones eg. progesterone (2) which is a carbon 21 hormone first synthesized during steroidogenesis pathway and regarded the primary progestational hormone produced by corpus luteum and placenta in some species of animals during pregnancy period which needs progesterone for maintenance (3).

Lutein cells in the corpus luteum have the capability to uptake and store cholesterol for synthesis and secretion of progesterone under the influence of lutenizing hormone LH (3). The most dramatic role of progesterone is that seen during pregnancy inhibiting myometrial activity and causes preparation for nidation, maintaining pregnancy by means of it's ability to inhibit cell- mediated responses involved in tissue rejection (3,4).

Furthermore, progesterone regulates hematological and biochemical levels during the pregnancy period with an enhancing female's body metabolism through this period, stimulates appetite and the tendency toward minimizing physical activity. The outline of these effects results in increasing maternal weight gain (1).

Rats and mice are prevailing as a biological model for researches related to pregnancy or teratogenic effects due to the relative short period of pregnancy (20- 23 days), in addition to numerous newborns (7-12) (5) with remarking that the three stages of pregnancy are resemble to those of other mammals (6).

Simvastatin is the most common drug that is affiliate to the statin family which are HMG Co- A reductase inhibitors with the net inhibition of mevalonate, so it's widely used for the treatment of hypercholesterolemia (7).

Available data thus far on the risk factors associated with cholesterol biosynthesis inhibition during pregnancy have need for a base study about these risks because some studies indicated that the inhibition of cholesterol biosynthesis caused a risky drop in the fertility parameters of male (8,9), rat females (10) and women (11) while other trails contracted these results showing no hazard effects (12,13).

The aim of this study is to inspect the maternal anomalies arising from cholesterol biosynthesis inhibition throughout pregnancy stages in the normal rats using a potent cholesterol inhibitor (simvastatin) and to evaluate it's detrimental effects related to serum biochemistry.

Materials and methods

Animals

Thirty mature female albino rats were obtained from lab animal house, College of Veterinary Medicine, University of Mosul. They were divided into two groups (fifteen rats each) maintained in a plastic cages (Kent Co. Ltd. England) under controlled environmental conditions (12:12 light:dark cycle, 23± 1 C°) with providing diet and water *ad libitum*.

Diet

A balanced basal diet was provided to the experimental animals and was formulated to meet their physiological needs during pregnancy period as recommended by the American Nutrient Research Council (14), diet prepared by manipulative mixing then compressed as a form of pellets, dried at 55 C° using hot air oven. To be assured the diet was free of cholesterol, Salkowski test was performed (15).

Drug

Simvastatin, SIMVOR®, 20 mg (RANBAXY laboratories Ltd. India) was used, in the form of suspension with deionized water which administered to rats by oral intubation.

Pregnancy diagnostic test

Three rat females were mated with an adult male rat in a separated cages at 4:00 pm., vaginal smears were obtained in the next early morning for microscopic detection of sperms which indicates the first day of pregnancy as stated by (16). Positive females were isolated and date recorded.

Experimental outlines

Two main groups included in this study; control group: comprised of fifteen rat females treated daily with deionized water by means of oral intubation since the first day of pregnancy along this period till 20th day. Simvastatin- treated group: comprised of fifteen females rat treated daily with simvastatin by means of oral intubation in a dose of 100 mg. kg^{-1} BW. (17) since the first day of pregnancy along this period till 20th day.

Specimens collection

Five rat females at the first day of pregnancy (just after giving positive pregnancy diagnostic test) were isolated for blood sampling (8:00- 9:00 am.) from retroocular vein (18) using capillary tubes to assess the base values of parameters. Blood was allowed to coagulate then centrifuged (1000 ×g at 4 C°) for 10 minutes, clear serum aspired by means of disposable Pasteur pipettes, kept in a polyethylene tubes in deep freezing. Then rats were sacrificed by separation of cervical spinal cord under light ether anesthesia, organs (liver, ovaries and placenta) were gently dissected, rinsed in ice- cooled isotonic saline (0.9% NaCl), dried on filter paper then kept in an aluminum foil in deep freezing.

At the end of the second stage of pregnancy (15th day), an additional five rats were sacrificed, while the remaining five rats were sacrificed at the end of third stage of pregnancy just before parturition (20th day) in the same manner. Also died fetuses, sizes and regressed was monitored.

Serum biochemical parameters

All biochemical parameters were achieved in the laboratories of the College of Veterinary Medicine, University of Mosul except the determination of progesterone which was carried out in Al- Bab Al- Sharqi clinical lab, Baghdad by means of radioimmunoassay using specific kit supplied from Immunotech Co., France.

Total cholesterol (TC), triglycerides (TG) and glucose were assayed by using of a specific photometric kits (Biolabo, SA. France) depends on the enzymatic principle. Alanine amino transferase (ALT) and aspartate amino transferase (AST), alkaline phosphatase (ALP) activities and albumin level were assayed using specific photometric kits (BioMerieux, SA. France). Total protein (TP) was assayed spectrophotometrically using Biuret method (15).

Tissue total cholesterol

Total cholesterol was extracted from the cells using chloroform: methanol mixture (2: 1 vol: vol) (19), chloroform phase was evaporated and the lipids redissolved by ethanol, TC was assayed in the ethanol containing lipids using specific photometric kit (20).

Analysis of data

The statistical significance of differences observed between the simvastatin- treated group and control was analyzed using student independent t- test by means of computer program, statistical package for social sciences (SPSS) (21).

Results

Significant decline (P≤0.01) in the serum TC concentration was clearly observed at the end of second and last stages of rat's pregnancy period respectively in the cholesterol biosynthesis inhibitor (simvastatin)- treated group relative to the control (figure 1), also TG concentration was significantly reduced (P≤0.05) at the second stage but more significant reduction (P≤0.01) observed at the 3d stage (20 days of simvastatin- treatment) (figure 2).

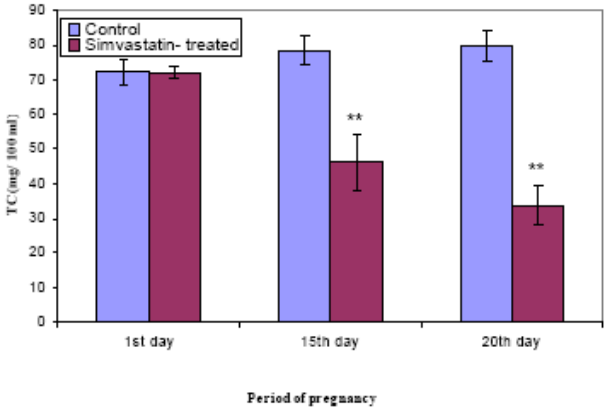

Figure 1: Effect of mevalonate biosynthesis inhibition on serum total cholesterol in normal pregnant rats.
** Significant at (P≤0.01), Values are expressed as mean± SE.

Serum progesterone was significantly (P≤0.05) reduced at the second stage with shifting toward more significant drop (P≤0.01) at the end of last stage (figure 3).

Serum glucose was significantly increased (P≤0.01) at both 2nd and 3d stages caused by the treatment with simvastatin keeping approach levels till the end of the 3d stage opposed with normal pregnant control (figure 4).

Data observed belongs to the concentration of serum albumin demonstrates a significant decrease (P≤0.01) in the simvastatin- treated group during the 2nd stage versus to control but values returns around that of control at the end of the last stage of pregnancy period (figure 5).

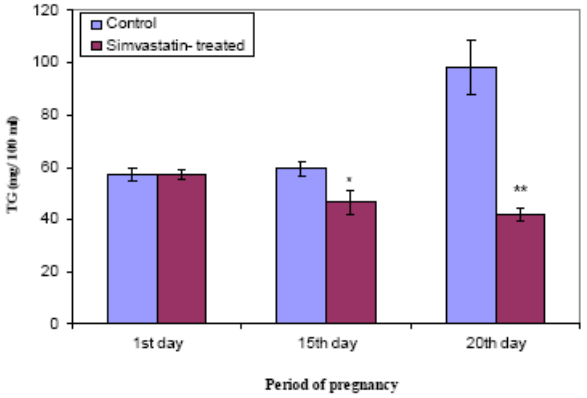

Figure 2: Effect of mevalonate biosynthesis inhibition on serum triglycerides in normal pregnant rats.
*Significant at (P≤0.05), ** Significant at (P≤0.01). Values are expressed as mean± SE.

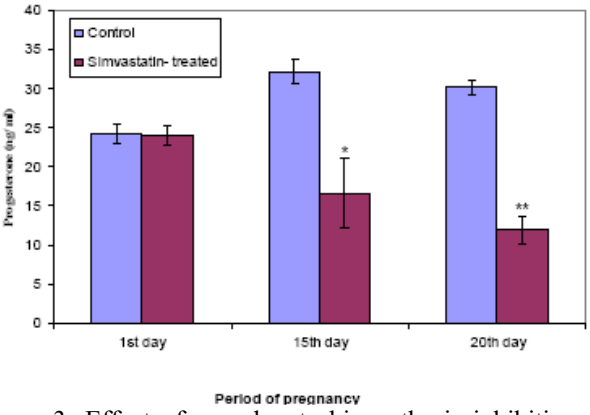

Figure 3: Effect of mevalonate biosynthesis inhibition on serum progesterone in normal pregnant rats.
*Significant at (P≤0.05), ** Significant at (P≤0.01). Values are expressed as mean± SE.

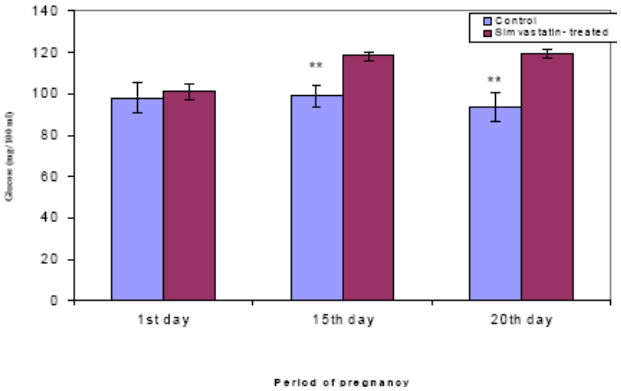

Figure 4: Effect of mevalonate biosynthesis inhibition on serum glucose in normal pregnant rats.
** Significant at (P≤0.01). Values are expressed as mean±SE.

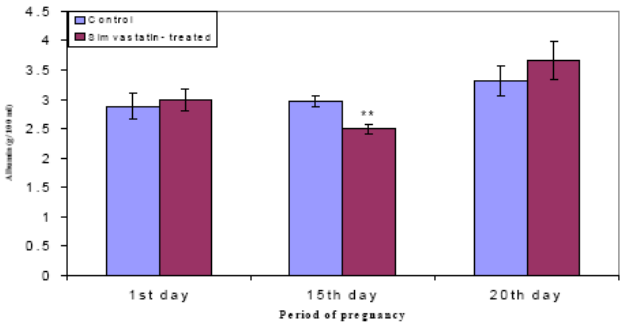

Figure 5: Effect of mevalonate biosynthesis inhibition on serum albumin in normal pregnant rats.
** Significant at (P≤0.01). Values are expressed as mean±SE.

On the other arm, the inhibition of mevalonate and consequential cholesterol biosynthesis did not alter serum TP concentration all over the duration of experiment (figure 6).

The activity of ALP was significantly elevated (P≤0.05) at the end of the 3d stage in the simvastatin-treated group compared with control (figure 7).

No change in the activity of AST was observed (figure 8) while the activity of ALT at the 2nd stage by the treatment with HMG Co-A reductase inhibitor was significantly elevated (P≤0.05) (figure 9) with return to homologue the value of control at the end of the 3d stage.

Data related to cholesterol concentration in the tissues demonstrated that there was a significant decline (P≤0.01) in the levels of total cholesterol in liver, ovary and placenta (table 1) of the simvastatin- treated group compared with control since the 2nd stage of pregnant rats treated with statins keeping on the same mode of drop in both 2nd and 3d stages on the same loom.

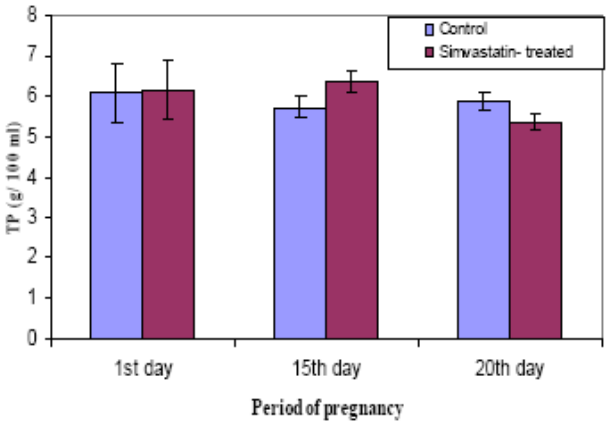

Figure 6: Effect of mevalonate biosynthesis inhibition on serum total protein in normal pregnant rats.
Values are expressed as mean± SE.

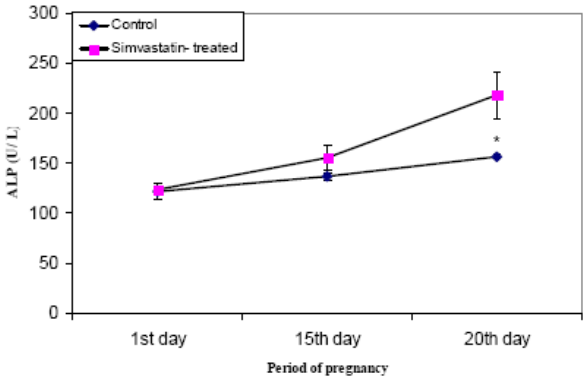

Figure 7: Effect of mevalonate biosynthesis inhibition on serum alkaline phosphatase activity progesterone in normal pregnant rats.
*Significant at (P≤0.05). Values are expressed as mean±SE.

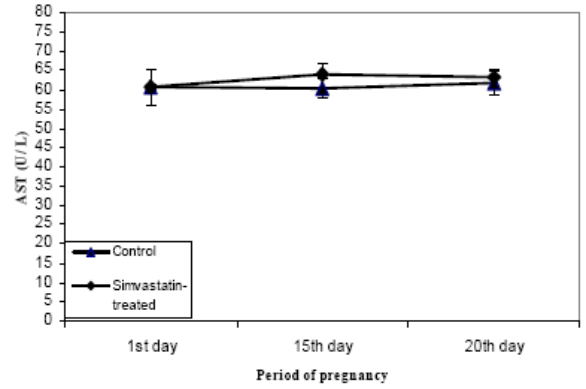

Figure 8: Effect of mevalonate biosynthesis inhibition on serum aspartate aminotransferase activity in normal pregnant rats.
Values are expressed as mean± SE.

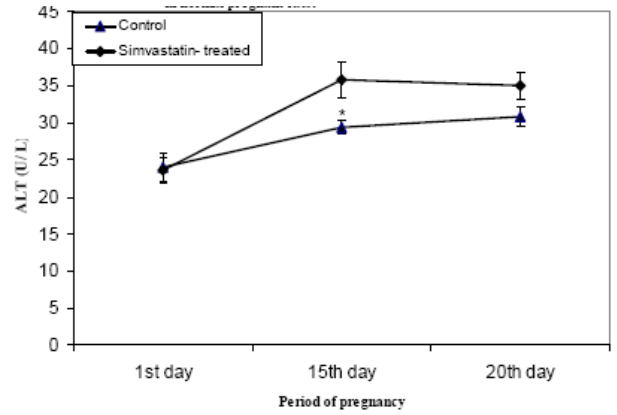

Figure 9: Effect of mevalonate biosynthesis inhibition on serum alanine aminotransferase activity in normal pregnant rats.
*Significant at (P≤0.05), Values are expressed as mean±SE.

Table 1: Effect of mevalonate biosynthesis inhibition on the concentration of cholesterol in the organs.

Organ	Group	TC (mg. g^{-1} wet tissue) Period of pregnancy		
		1st stage (1st day)	2nd stage (15th day)	3d stage (20th day)
Liver	Control	38.40± 2.32	54.22± 4.15	46.32± 1.89
	Simvastatin - treated	36.93± 1.55	18.50± 2.44**	13.93± 4.01**
Ovary	Control	70.42± 2.48	91.66± 2.83	73.56± 1.95
	Simvastatin - treated	72.56± 1.40	49.14± 4.09**	44.56± 2.95**
Placenta	Control	34.60± 1.07	35.06± 2.0	35.78± 1.61
	Simvastatin - treated	33.72± 1.12	23.31± 1.69**	13.50± 2.48**

** Significant at (P≤0.01), Values are expressed as mean± SE.

Worthy mentionable that all simvastatin- treated pregnant rats which sacrificed at the 20th day of treatment were undergone from no living embryos detected in the uterine section, decreased number, size with evidence of increased regressed embryos.

Discussion

An adequate pool of intracellular cholesterol as well as mevalonate and it's isoprenoid derivatives is essential for steroidogenesis in gonads providing an important contributions to the maternal animal during pregnancy and lactation (22,23), therefore the inhibition of cholesterol biosynthesis and subsequent mevalonic acid will deprives the body from these essential precursors for promotion of steroidogenesis (24). Statins were among the most common inhibitors of *de novo* cholesterol biosynthesis, so they are used prescription medications world wide (25) in order to reduce serum cholesterol but malevolence these medications may have a hazard effects especially those related to steroid hormones biosynthesis (24).

It is an axiomatic result to detect a reduction in serum TC caused by mevalonate biosynthesis inhibition by means of simvastatin treatment, data which confirmed the previous studies in rabbit's females (26), women (27) and in normal male rats (24). It was mainly correlated with the inhibitory potency of statins toward cholesterol biosynthesis (28), namely inhibitory activity toward mevalonate biosynthesis followed by reduction of downstream cholesterol and yielding metabolites in the pathway (29) as well as the

ability of statins to enhance mRNA of low density lipoproteins receptors by an action of serum cholesterol level on the sterol regulating element- binding protein which controls the specific gene expression participating in the cholesterol cellular uptake mechanism and metabolism leading to a drop in it's plasma level (2). Results in this study were fully agree with the previously discussed results related to TC in normocholesterolemic rats (30,31).

The decline in serum TG is harmonizes with (31) in normal rats submitted to cholesterol biosynthesis inhibition using simvastatin irrespectively to pregnancy, the condition which also considered a key factor in dropping of plasma TG in the rats which is refer to the suppression of cytosol facing microsomal enzyme, diacylglycerol acyltransferase activity which in turns to catalyze the final step in TG biosynthesis, an event which defendant in the declining of TG level (32) beside a supplementary explanation suggesting a different mechanisms may be involved in the hypotriglyceridemic effect of statins despite the induction of the key enzyme involved in very low density lipoproteins- cholesterol VLDL-c production (33).

The decrease in progesterone level throughout the experiment is resemble to those studies in human placental explants (11,28), which they used different inhibitors of cholesterol biosynthesis, the strong inhibition of sterol synthesis significantly attenuated the secretion of progesterone although there was an evidence that statin treatment does not impair the secretion of progesterone and other steroids hormones (13). Also the inhibition of cholesterol biosynthesis had no influence on progesterone secretion into the culture medium which was probably due to the presence of large cholesterol pools (34). HMG Co-A reductase inhibitors in addition to their well pronounced mechanisms in enzyme- competitive inhibition, limiting the availability of the sterol precursor, they have the ability to reduce receptor- mediated up take of cholesterol for steroidogenesis (35). Also the treatment with statins results in proliferation inhibition of theca interstitial cells DNA (36) through aborting of isoprenalation process for GTPase, so inhibits progesterone secretion (10), a recent study referred to further molecular mechanism by which HMG Co-A reductase inhibitors exerts their effects in rat's ovary through down regulation of lutenizing hormone LH-receptors with a net inactivation of fibroblast growth factor-9 which practices a role in enhancing steroidogenic acute regulatory protein and P450 side chain cleavage mRNA level, an observation providing a mechanism of action for the decreased progesterone secretion (37). Furthermore an orphan mice nuclear receptor NRsA2 involved in cholesterol metabolism and embryogenesis may negatively affected by cholesterol biosynthesis inhibition leading to an astriction to progesterone biosynthesis (38).

One of the gestational features in the rats is lower concentration of serum glucose compared with non pregnants (39,40), on the other arm, statins- treated normal non pregnant rat females recited in a decline in serum glucose concentration (41), the same findings were observed in hyperglycemic male rats from protein-restricted dams (42). Progesterone, the dominant hormone during pregnancy in all species had been proven to enhance insulin secretion as well as it up regulates insulin receptors (43) so the restriction in progesterone level in this critical duration (pregnancy) may overcame on the hypoglycemic effect of statins and considered a vital prompt for development of insulin resistance and subsequent hyperglycemia (44) which are fit with our observations in simvastatin- treated pregnant rats.

Previous studies revealed that the inhibition of mevalonate biosynthesis by HMG Co-A reductase inhibitors decreased serum albumin in normal biological models (45,46), also investigators demonstrated that the physiological conditions accompanying pregnancy may be the suspect reason in decreasing serum albumin in rat females (39,40) non mentioned about the combination effects of statin- related compounds on one hand and pregnancy on the other hand related with serum albumin.

The present study showed a decrease in serum albumin concentration till the end of 2nd stage of pregnancy in the simvastatin- treated group compared with control an observation which can given an interpretation through podocytes- mediated endocytosis of albumin in statin-sensitive manner (47) however it returns to homologue the value of untreated control at the end of pregnancy period which elucidated the role of progesterone which notably inhibited in the adjustment of some biochemical values during this critical period (43), also this event is partially due to the increased albumin retention in the body during simvastatin influence (48) in spite of that albumin represents the greater ratio of TP, the last did not show differences between the normal pregnant control and simvastatin- treated which antagonized some results of (49).

No alteration in ALP activity caused by simvastatin oral dose till the end of 2nd stage which assured the observations of (50), however ALP activity elevation was observed at the end of 3d stage by mevalonate biosynthesis inhibitor suggesting a severe placental damage caused by progesterone restriction leading to elevated ALP activity due to elevated placental isoenzyme of ALP (51).

The activity of AST did not show significant changes during the treatment with simvastatin, a result to be against of each (24) in male rats and in vitro study of (52) which they found that statin- related compounds may elevates plasma AST activity, on the other arm ALT activity revealed an increase at the end of 2nd stage of pregnancy in comparison with control plus resumption to the near value of control at the end of 3d stage which is in fully agreement

with (53,54) who explains that statins improved liver enzymes after exposure to liver failure and damage in rats.

Data belongs to liver TC are zonal in the progressive high significant decline reaching about one third of the value of control because liver TC level is directly related to serum TC depending on lipoprotein transporting mechanism (2). These observations are agree with those explained by (24) in normal male rats and with (55) who concluded a proximate relationship between liver TC and liver activity of HMG Co-A reductase, furthermore it was found that the treatment with HMG Co-A reductase inhibitors may induce an expression of sterol regulatory element binding protein- target genes, causing different effects attributed to reduced liver TC (33).

Ovarian concentration of TC was reduced on the same extent of that observed in liver with regard to high level of ovarian cholesterol production which resembles results of (56), this is not only associated with inhibition of HMG Co-A reductase activity but another mechanisms had been proven in rat's ovarian cells which cited that the inhibition of mevalonate pathway may induces a concentration-dependent inhibition of theca interstitial cell proliferation (10), also changes in expression levels of periovulatory granulosa were detected for more than eighty genes, a sub set of them are involved in cholesterol homeostasis forming a functional cluster as well as cholesterol synthesis correlates closely with the level of mitochondrial HMG Co-A synthase which was among the down regulated genes (28).

Related demonstration strengthening these mechanisms what documented about HMG Co-A inhibitors which increased the degree of periovulatory granulosa apoptosis supporting this idea through inhibiting protein isoprenylation with the observed decrease in the expression of several genes in the cholesterol biosynthesis pathway including enzymes acting down stream from mevalonate. This explains the ability of mevalonate to reverse the apoptotic effect of statins (57).

As in the liver and ovary, placental cholesterol showed remarked inhibition, a result was similar with outcomes of (58) although there was an evidence that the rate of cholesterol biosynthesis is lower in dams in comparison with embryo (30).

Previously stated results, diminished embryos size and increased the regressed fetuses were recorded by inhibition of mevalonate biosynthesis and subsequent limiting of progesterone secretion although some studies demonstrated that statins concentration in the reproductive organs was 50% of that in the maternal blood (59) with no detection of still births in pregnant women (60). Simvastatin- related compounds have the potential to inhibit fibroblast migration with tendency to induce and promote apoptosis in cytotrophoblasts which impairs implantation in addition to the dominant effect in decreasing progesterone (11).

It can be concluded from this study that the inhibition of mevalonate biosynthesis and related isoprenoids including cholesterol during gestation of rats perpetrates a deleterious effect on progesterone production and some biochemical parameters with an articulate depletion for cholesterol conserved in vital organs which may leads to failure in pregnancy, also asserted the vital role of progesterone in organizing the serum biochemistry during this period.

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