

# Hypercholesterolemia role of vitamin D3 and genistein effect in reducing atherosclerosis in rat

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## ABSTRACT

**Introduction:** Atherosclerosis has been identified as the most agents for premature death and disability and of its injuries is growing masses of fatty materials with fibrous connective tissue and deposition of calcium salts and other blood materials. To do research on atherosclerosis, in creating hypercholesterolemia animal model is important.

**Methods:** In this wxperimental study, 4 groups of rats were used (n=12). Group 1 and Group 2 received food-based dietary and Group 2 addition of it, received 2% cholesterol and 0.5% colic acid. Groups 3 and 4 in addition to the group 2 food, respectively. Vitamin D 3 (300000IU/Kg BW) and vitamin D2 (15000IU/Kg BW), received group 3 had the highest cholesterol levels after 6 weeks that were distributed to 3 subtypes A, B and C. A subgroup received previous diet, and subtypes B and C, in addition to the previous feed, respectively received genistein with doses 1.5 and 3 mg/kg BW for 6 weeks. For statistical analysis of the data ANOVA and LSD tests were used and  $P < 0.05$  was considered significant.

**Results:** In the first stage there were significant differences in cholesterol, LDL, LDL/HDL and atherogenic index between experimental and control groups and also between group 3 and other two treatment groups that is the effect of vitamin D3 for hypercholesterolemia model. The Second stage, revealed the effect of genistein to reduce the cardiovascular damage.

**Conclusion:** Vitamin D3 is one of atherogenic factors that if it be accompanied with high cholesterol, can be caused atherosclerosis similar to human that can be used for studying related to atherosclerosis. Types of isoflavones in diets can reduce atherosclerotic damages due of vitamin D3 accompanied with high cholesterol.

**Key words:** Rat, Atheosclerosis, Vitamine D3, Vitamine D2

## Introduction:

Atherosclerotic disease that is known as the most important factors of premature death and disability (1), is Multifocal and its damages are formation the growing masses of fatty material associated with the fibrous connective tissue and

precipitated calcium salts and blood cells such as macrophages and lymphocytes (2). To carry out research on atherosclerosis, the creating of hypercholesterolemic model in animals is very important. The creation of this model is not easy, especially in rats (3,4). High-cholesterol diet in rats

causes an increase in apolipoprotein E gene expression by the liver cells that increases the reverse transition of LDL into liver cells (5) and LDL breaks down by lysosomes. Ester and Apo Cholesterol 100-B decomposed and then the cholesterol will be used for the synthesis of cell membranes, steroid hormones and bile acids. On the other hand, intracellular cholesterol increase inhibits the HMG-CoA reductase enzyme and prevents making cholesterol from acetyl coenzyme A (6,7). Therefore, increasing levels of cholesterol in rats hardly takes place. Using specific doses of vitamin D in combination with cholesterol-rich diet can create complex damages such as lipid necrotic pores, fibrotic cap (8-10). Therefore, the aim of the first step in this study was to investigate the possible use of vitamin D with a high-cholesterol diet for creating hypercholesterolemic model in rats.

The results have shown that soy purified isoflavones may have an effect on the damaged arteries without changing the levels of lipid and lipoprotein (11). Isoflavones, in terms of chemical structure and function, between phytoestrogens are most similar to estrogen (12). Of the distinctive features of phytoestrogens is the compatibility and increase their estrogenic activity during low estrogen or reducing estrogen activity (11). While the therapy is raised with estrogen, isoflavones can be used as an alternative. Isoflavones connections with estrogen receptors are widely varied. Genistein binding amount of globulin that connects to sex hormone is ten times lower than estradiol (13,14).

Therefore, the free Genistein concentration is relatively high in plasma and its tendency to bind with estrogen receptor increases.  $\beta$  Estrogen receptor plays a role in maintaining the normal operation of vessels (4). Experimental evidence in rats suggests that to improve mechanical damage to the arteries, Genistein applies its protective effect by  $\beta$  estrogen receptor of arterial smooth muscle cells (15,16). In summary, the usefulness of Phytoestrogens is by reducing the oxidation of lipids, cholesterol stability settings, prevention of clotting and blocking the expression of inflammatory cytokines that are the causes of cardiovascular disease (1,17-20). Therefore, the aim of the second phase of the study was the investigation of Genistein role in reducing coronary

and aortic artery lesions in creating hypercholesterolemic model in rats.

## Methods:

48 male Wistar rats weighing  $20 \pm 200$ g were prepared and maintained in the same condition for two weeks and after compliance with animals' nest in Isfahan University; they were distributed into four groups of duodecimal. A basic diet consisted of 20% protein, 50% starch, 10% cellulose, 15% fat and essential vitamins in the standard limit.

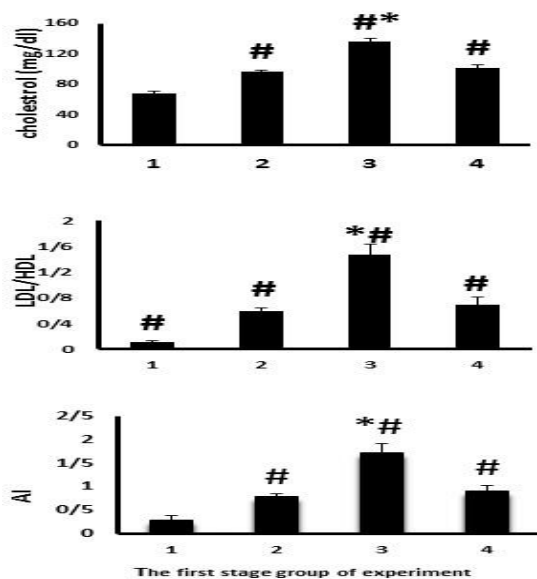
In the first stage of the experiment, the first group of rats as a control group received basic diet. The second group along with basic diet received 2% cholesterol and 0.5% colic acid. The third and fourth groups in addition to receive diet same as the second group, daily by intramuscular injection, they received respectively vitamin D3 with 300000 dose IU/kgBW (21,22) and vitaminD2 with 15000 doses (23) IU kgBW. After six weeks, blood samples were taken and the amount of cholesterol, triglyceride and HDL in serum was measured and LDL was calculated by the formula (total cholesterol - (triglycerides / 5 + (HDL) and the third group that the cholesterol level, LDL and LDL/HDL ratio were at the highest level, was considered as hypercholesterolemic model for the second phase of the research.

The second phase of the study lasted for 6 weeks, so that the group 3 (12 rats) that had the highest serum cholesterol, were divided into three groups of A, B, C. For the subgroup A as the control group, the same cholesterol-rich diet with vitamin D3 in the first stage was continued. Subgroups of B and C in addition to similar diet of subgroup A, treated with Genistein, by gavage respectively received 1.5 mg/kgBW and 3 mg/kgBW. At the end of the second stage, the blood samples were taken and all rats were sacrificed with pentobarbital solution and were sampled for the detection of tissue damages from coronary arteries and aorta. The heart samples were isolated with aortic and after washing with 9% physiological serum, they were kept in 10% formalin. Coronary and aorta arteries samples were isolated from each heart; they were kept in identifying containers with specified label in 10% formalin at a rate of 10 times of the tissue volume.

Histology blocks were prepared from the heart and aorta of each rat. Consecutive sections were prepared from each block in 2 to 4 microns thick and after the establishment of the sections on a microscopic slide; they were dyed with hematoxylin and eosin. Microscopic examination of tissue samples was done with 10X, 40X and 100 X magnification. Damages in the intima were identified and the relative size of plaque to evaluate the coronary and aortic arteries pathological examination was calculated. Statistical data analysis was performed by using SPSS version 16 software. The Lipids and serum level of lipoproteins as well as the index of atherogenic (VLDL+LDL)/HDL between the groups were done in both first and second phases of the study by using one-way ANOVA and LSD test and the amount of  $P < 0.05$  was considered significant.

## Results:

At the end of the first phase, the cholesterol, LDL level and LDL/HDL ratio in the serum of all groups was higher than the control group ( $P < 0.05$ ). In addition, the mentioned blood factors in the third group that received the cholesterol-rich diet with vitamin D3 compared with the high cholesterol group and with high cholesterol with the vitamin D2 group was significantly higher ( $P < 0.05$ ).



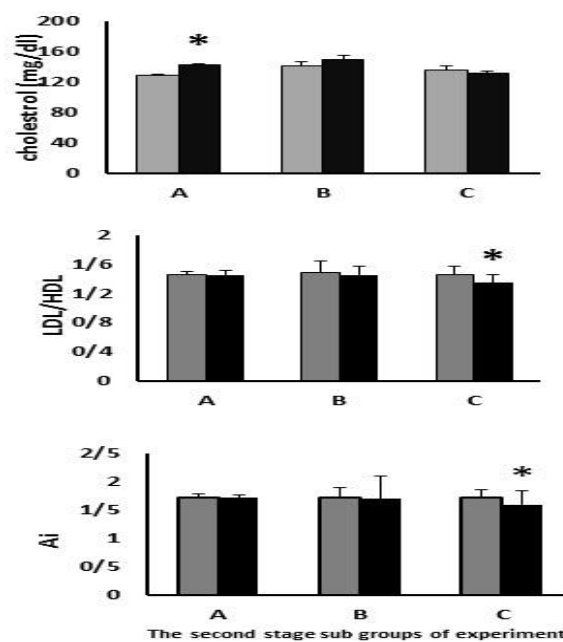
**Figure 1. Comparison of blood factors in the first stage**  
\*Significant, compared with other group

# Significant compared with control group

First stage: group1: Basic diet- group2: Basic + hyper cholesterol diet  
group3: basic + hyper cholesterol diet+ Vita D3  
group4: basic + hyper cholesterol diet+ Vita D2

This difference suggests that the use of cholesterol-rich diet with vitamin D3 can create a hypercholesterolemic model in rats (Figure 1).

At the end of the second phase, the cholesterol, LDL level and LDL/HDL ratio in the serum of subgroup C was significantly ( $P < 0.05$ ) less than subgroup A, but it had no significant difference with subgroup B that received 5.1mg/kgBW Genistein. This implies the positive effect of Genistein in preventing the cholesterol, LDL level and LDL/HDL ratio increase in animals receiving cholesterol-rich diet along with vitamins D3 (Figure 2).



**Figure 2 - Comparison of blood factors in the second phase with the first stage.**

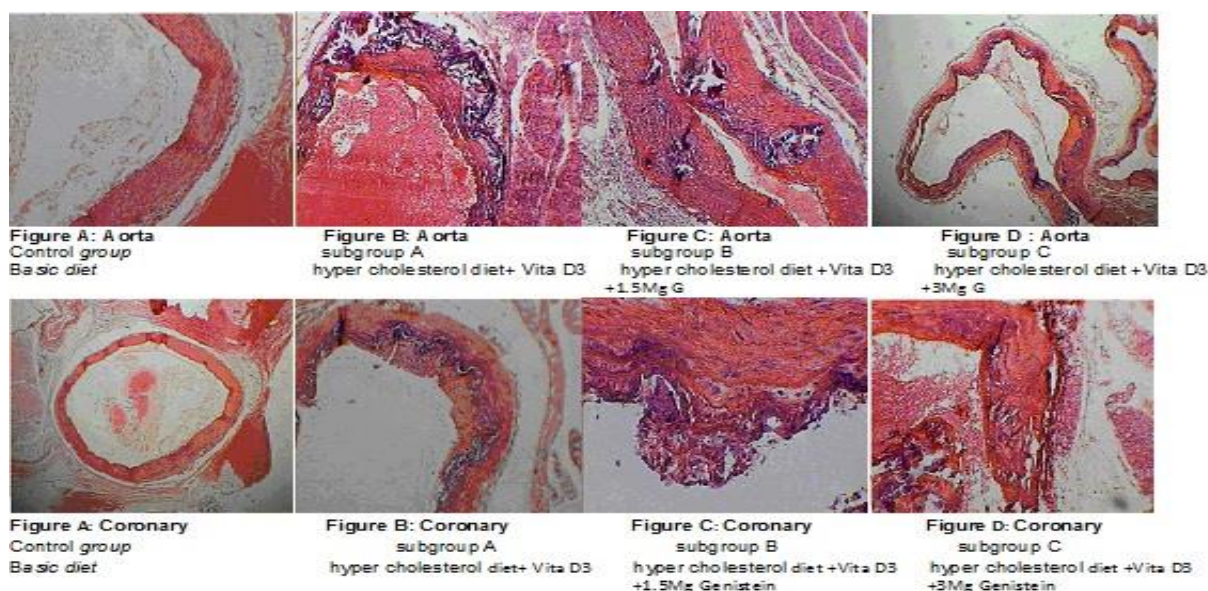
Gray columns represents the first stage and black column represents Second stage

\* Significant, compared to the first stage (A, B, C: D3+ hyper cholesterol)

Second stage (subgroup: hyper cholesterol diet+ Vita D3-  
subgroup B: hyper cholesterol diet + Vita D3 + 1.5Mg Genistein-  
subgroup C: hyper cholesterol diet + Vita D3+3Mg Genistein

The average size of atherosclerotic plaques in the aorta of rats subgroup A (vitamin D3+high cholesterol) was equal to 41.6% and in subgroup B, receiving the same diet as subtypes A with

Genistein (1.5 mg/kgBW) was equal to 18.30% and in the subgroup C, receiving the same diet AS subgroup A with Genistein (3mg/kgBW) was equal to 16.6 percent (Figures a, b, c, d). The average relative size of atherosclerotic plaques in coronary, or in other words, the percentage of atherosclerotic lesions of blood vessels in subgroup A was determined 51.6%, in the subgroup B was 20% and in the subgroup C was 15%, respectively (Figures e, f, g, h) . These results indicate that the animals in subgroup C were less involved in vascular injury. In other words, this situation reflects the positive and increasing effect of Genistein in preventing the atherosclerotic plaque formation in coronary and aorta arteries.



## Conclusion:

This study was designed in such a way that the role of vitamin D3 in creating hypercholesterolemic model and Genistein effect in reducing the accumulation of fat in the coronary and the aorta veins in rats were investigated. While creating an animal model of atherosclerosis in hypercholesterolemic research is very important, rats are not susceptible to progression of atheroma in response to cholesterol-rich diet. The results showed that prescribing vitamin D3 with cholesterol-rich diet could be effective in creating hypercholesterolemic model in rats and causing the

atherosclerosis almost identified with its advanced kind in the humanities.

In addition, the amount of damage in the coronary and the aorta arteries in rats that were received cholesterol-rich diet along with vitamin D3 and were treated with Genistein, compared with rats that only were received vitamin D3 with high cholesterol diet, significantly decreased. Although, in the second stage of the research, the increasing and the positive effect of Genistein was observed in preventing the atherosclerotic plaque formation in the coronary, but there was not a statistically significant difference between the effects of low-dose and a double dose of Genistein. This indicates

that if the effect of Genistein dose is concerned with the prevention of atherosclerosis, it is required to have a considerable difference between low dose and high dose, for example, about four times.

Calcification caused by media in groups receiving cholesterol-rich diet with vitamin D3 is consistent with reported results of Fleckenstein and colleagues and Rajasree (24,25). The mechanism for these effects of vitamin D3 would be that the use of high-dose of this vitamin increases the serum calcium in the first few days (26,27). Then an increase in intracellular calcium levels causes smooth muscle cells destroy, serine protease enzyme releases and Osteopontin gene expression. Serine protease enzyme causes the elastin fibers into pieces and tissue Osteopontin and calcification processes (24,28) follow it. Another mechanism by Rajasree and colleagues is based on that the use of high dose vitamin D3 reduces parathyroid hormone and increases the alkaline phosphatase activity, which leads to calcification (16,25). Jono and colleagues with a study on the culture medium (29) confirm this mechanism. another proposed mechanism of Rajasree is that the use of high dose vitamin D3 increases the gene expression of receptors related to D3 and  $(OH)_2$  25 and 1 in aortic smooth muscle cells and thus the hormone complex and receptor increase intracellular calcium and calcified the smooth muscle cells (25,30).

In some studies, vitamin D3 was used without a cholesterol-rich diet, so only calcification was observed in the aorta and coronary artery Media (15). Given that vitamin D3 was used in combination with cholesterol-rich diet in this study, not only calcification was observed in media, but also the fibrous parts were in intima that is almost like advanced atherosclerosis in humans. Fibrous plaques are because of the growth factors release and macrophages and platelets development, endothelial cells and smooth muscle cells in the intima. With this process, this unusual type of synthesis feature in smooth muscle overcomes its contraction nature and causes smooth muscle cells to proliferate and accumulate. Smooth muscle cells become foam cells with absorbing OX-LDL and by increasing the synthesis of extracellular matrix contribute to the formation of fibrous plaques (31,32). Therefore, Vitamin D3 with cholesterol-

rich diet facilitates the progress and development of plaque in hypercholesterolemic model in rats.

The results of this study confirm the important fact that isoflavones may play a deterrent role in the progress and development of atherosclerosis plaque. Different mechanisms are provided for preventing the effects of isoflavones. Antony et al (2000) reported a net soy isoflavones inhibits the processes such as tyrosine kinase activity, the production of proinflammatory cytokines; migration and proliferation of smooth muscle cells, platelet aggregation and oxidation of LDL, it prevents complications of atherosclerosis (1). Many reports confirmed these mechanisms based on the antioxidant properties of isoflavones (33,34).

Research results of Makela et al. (1999), Zhu, and colleagues (2002) have shown that Genistein applies the effect of preventing the proliferation of smooth muscle cells in response to mechanical damage suffered by the vessel in rats, via  $\beta$  receptor (35).

The research results of Clarkson et al (2002) showed that a diet rich in soy proteins in capable rats, prevents atherosclerosis, and if soybean isoflavones is removed by ethanol, these effects are reduced. The mentioned effects are independent of the presence or absence of LDL receptors and the effects on plasma lipids and lipoproteins (36,37).

Because isoflavones are similar to the estrogen structure, so their anti-atherosclerotic role can be played through the mechanism similar to estrogen. The role of estrogen in preventing atherosclerosis is played by preventing the expression of MCP-1 genes; creating adhesion molecules and reduction of pre-inflammatory cytokines such as IL-6 (38). One of the progress stages of atherosclerosis is the proliferation and migration of smooth muscle cells. A number of researchers have reported that Genistein prevents proliferation and migration of smooth muscle cells by changing the growth factor activity (39). It was also reported that isoflavones improves the damaged endothelium and reduce aggregation and the activity of platelet (39,40).

Although, creating the hypercholesterolemic model in rats is not easy, but vitamin D3 with high cholesterol diet can be used as an atherogenic factor to carry out researches on atherosclerosis.

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## نقش هیپرکلسترولمیک ویتامین D3 و اثر ژنیستین در کاهش آترواسکلروز در رت

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### چکیده

**مقدمه:** بیماری آترواسکلروز که از مهمترین عوامل مرگ و ناتوانی زودرس شناخته شده و از آسیب‌های آن تشکیل توده‌های رو به افزایش مواد چربی مرتبط با بافت همبند فیبروزی و رسوب نمک‌های کلسیم و سایر مواد خونی است. برای انجام پژوهش‌ها در زمینه آترواسکلروز، ایجاد مدل هیپرکلسترولمیک در حیوانات بسیار حائز اهمیت است.

**روش کار:** از ۴ گروه نوازده‌تایی رت استفاده شد. گروه ۱ خوراک پایه و گروه ۲ افزون بر خوراک پایه ۲ درصد کلسترول و ۵/۵ درصد اسید کولیک داشت. گروه‌های ۳ و ۴ علاوه بر خوراک مشابه گروه ۲، ویتامین D3 به میزان ۳۰۰۰۰ IU/kgBW و ویتامین D2 به میزان ۱۵۰۰۰ IU/kgBW دریافت نمودند. ۶ هفته بعد در انتهای مرحله اول گروه ۳ که بالاترین کلسترول سرم را داشت، به ۳ زیرگروه A، B و C توزیع شد. زیرگروه A خوراک قلیی و زیرگروه‌های B و C علاوه بر خوراک قلیی به ترتیب دوزهای ۱/۵ mg/kgBW و ۳ ژنیستین را به مدت ۶ هفته دریافت کردند. برای تجزیه و تحلیل آماری داده‌ها در هر دو مرحله اول و دوم پژوهش از آنالیز واریانس یک طرفه و سپس آزمون LSD استفاده شد و مقدار  $P < 0.05$  معنی‌دار تلقی گردید.

**نتایج:** مرحله اول، کلسترول، LDL، HDL، LDL/HDL و اندیس آتروژنیک بین گروه‌های آزمایشی و گروه شاهد و بین گروه ۳ و دو گروه تیمار دیگر تفاوت‌های معنی‌دار وجود داشت که نمود اثر ویتامین D3 برای مدل هیپرکلسترولمیک است. مرحله دوم، اثر ژنیستین در کاهش عروق قلبی را نشان داد.

**نتیجه‌گیری:** ویتامین D3 یکی از عوامل آتروژنیک است که اگر با خوراک پرکلسترول همراه شود می‌تواند در رت آترواسکلروز مشابه انسان ایجاد نماید که برای انجام پژوهش‌ها در زمینه آترواسکلروز می‌توان از آن استفاده نمود. انواع ایزوفلاون‌ها در رژیم‌های غذایی می‌توانند آسیب‌های آترواسکلروتیک ناشی از وجود ویتامین D3 همراه با رژیم پرکلسترول را کاهش دهند.

**کلیدواژه‌ها:** رت، آترواسکلروز، ویتامین D3 و ویتامین D2

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