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Evaluation of the Effect of Silymarin Alone and in Combination with Calcium on Bone Formation, Resorbtion and Turn over in *Ovariohysterectomized* Rat Model

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ABSTRACT

Background: Osteoporosis represents a bone mass loss unassociated with other chronic diseases and is related to estrogen deficiency and aging. Bone status can be described by measuring bone mineral density. Silymarin is a purified extract from milk thistle (Silybum marianun). Estrogenic effects of SIL have been observed in ovariectomized (OVX) rats. To evaluate the effect of Silymarin alone and in combination with calcium on bone formation, reabsorbtion and turnover, ninety virgin female rats (120–150g) aged twelve weeks, were randomized into nine groups of ten rats each. Eight groups were ovariohysterectomized under standard anesthesia and one group left intact as an un-operated-control group. After surgery 3 operated groups received silymarin, calcium and combination of both from day five post-op and 3 operated groups received the same treatment 4 weeks after surgery; two operated groups were considered as sham and control operated groups. Treatment continued for 4 weeks in each group. On the 0, 30 and 60 post operation day blood samples were obtained and Osteocalcin, Osteonectin, crosslaps, Serum estradiol (E2) and progesterone were measured. Silymarin alone and in combination with calcium did not alter elevated serum estrogen, progesterone and osteocalcin concentration but could decrease elevated serum osteonectin and crosslaps concentration after oral administration. The current report demonstrated that silymarin is a preventative agent when it is combined with calcium against osteoporosis and this treatment may dictate its effect via bone resorption processes. Each of the treatments, silymarin or calcium, has their own independent way to compromise osteoclastic bone resorption.

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INTRODUCTION

Bone, as a structural tissue, is subject to stress damage that can eventually lead to fracture if allowed to progress. However, bone has the ability to repair itself by a process called “bone remodeling” which consists of the removal of older bone tissue (resorption) and replacement with new bone tissue (formation). Bone resorption is performed by osteoclasts whereas synthesis is performed by osteoblasts, and an imbalance in this process can lead to disease states such as osteoporosis, [1]. Osteogenesis is a strictly regulated developmental process, in which numerous hormones and growth factors activate osteoblastspecific signaling proteins and transcription factors required for osteoblast differentiation. Osteoblasts are responsible for the mineralization of extracellular matrix (ECM), the terminal step of osteoblast differentiation [2, 3]. The constant process of bone remodeling consists of four consecutive stages [2].bone resorption, during which osteoclasts omit old bone; reversal, in which mononuclear cells appear on the bone surface; bone formation, in which osteoblasts completely replace resorbed bone with new bone; and bone mineralization, during which osteocytes are embedded within the bone matrix. Osteoporosis represents a bone mass loss unassociated with other chronic diseases and is related to an estrogen deficiency (Type I) and aging (Type II) [3]. Older aged women reach the stage were the ovarian function ceases. These causes a decline in the estrogen concentrations in the body.Many studies have shown that estrogen intervention reduces the rate of bone loss among postmenopausal women [4, 5, 6, 7]. Bone status can be described by measuring bone mineral density, which provides information on bone mineral content. Bone formation that exceeds or lags resorption is obtained, qualitatively, by measuring biochemical bone markers that are produced or released during bone turnover. Biochemical techniques have been developed to measure

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products of bone resorption and bone formation, and thus the degree of bone turnover can be measured. Osteocalcin is one of the bone formation markers. This is a major noncollagen protein of the bone matrix. Osteocalcin is vitamin K dependant and is synthesized in osteoblasts and megakaryocytes [8].

Collagen type 1 Telopeptides or crosslaps are another marker that reflect bone resorption. Collagen type I break down is mediated by acid proteases. This is an enzyme derived from osteoclasts. This marker shows changes in osteoclastic activity. The goal of an animal model is to replicate the human condition as closely as possible. Rodents provide the most commonly used model for the study of osteoporosis. Silymarin (SIL) is a purified extract from milk thistle (Silybum marianun), composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin and silychristin [9]. Silymarin and silybin used so far mostly as hepatoprotectants were shown to have other interesting activities [10]. Estrogenic effects of SIL have been observed in ovariectomized (OVX) rats [11, 12].Many drugs such as Kaempferol exerts and Dried plum polyphenols have been shown to have anti-osteoclastogenic effects [13, 14]. It seems that the inhibition of osteoclastogenesis by these compounds may be partially attributed to their anti-inflammatory and antioxidant properties. Accordingly, natural compounds and dietary components with antioxidant and anti-inflammatory activity may optimize bone health and stimulate bone formation. Silibinin is known to be an effective bioactive antioxidant, conserving glutathione in live cells while stabilizing the cell membranes against oxidative attack [15]. The goal of any alternative treatment is to find ways of managing diseases or healing without the use of drugs. Some alternative treatments and therapies may be used for osteoporosis. There is little scientific or clinical evidence to suggest that these therapies are truly effective. However, many people claim success with such treatments. While more scientific research is needed on the subject, some herbs and supplements are believed to reduce or potentially stop bone loss caused by osteoporosis. Since herbal medicine has become popular over the last decade and osteoporosis is responsible for considerable morbidity as well as deaths in many older women [16]. The aim of this study is to evaluate of the effect of Silymarin alone and in combination with Calcium on bone formation, reabsorbtion and turnover in *ovariohysterectomized* rat mode.

MATERIALS AND METHODS

Silymarin:

Silymarin purchased from Sigma, USA, contained the following substances: about 70% silybinin and dehydrosilybin, 14% silydianin and 16% silychristine. All other chemicals used were of analytical grade.

Animals and treatment:

Ninety virgin female rats (120–150g) aged twelve weeks were randomized into eight groups of ten rats each. They were housed under controlled environmental conditions (12:12-h light/dark cycle) and had free access to standard pellet chow and water. Seven groups were ovariohysterectomized (OVX) under standard Ketamine and xylazine anesthesia, one group was control-operated and one group left intact as an unoperated-control group. On the following two consecutive days, each ovariohysterectomized animal received 10000 IU/kg penicillin, and 20-50mg/kg Metamizol. Meloxicam 0.4mg/kg treatment was performed after the operation. Sutures were removed ten days after the intervention. After surgery the rats in the treatment groups were treated as follows:

- (OVX I) Oral administration of silymarin (SIL; 50 mg/kg/day) [12] from fifth postoperative day for 60 days.
- (OVX II) Oral administration of Calcium (250 mg/kg body weight/day, corresponding to 0.5% calcium content) [17,18], from fifth postoperative day for 60 days.
- (OVX III) Oral administration of silymarin (SIL; 50 mg/kg/day) and Calcium (250mg /kg body weight/day) from fifth postoperative day for 60 days.
- (OVX IV) Oral administration of silymarin (SIL; 50 mg/kg/day) from 30 days after operation for 30 days.
- (OVX V) Oral administration of Calcium (250 mg/kg body weight/day) from 30 days after operation for 30 days.
- (OVX VI) Oral administration of silymarin (SIL; 50 mg/kg/day) and Calcium (250 mg/kg body weight/day) from 30 days after operation for 30 days.

Collection of blood samples:

On days 0, 30 and 60 post-op blood samples were obtained from dorsal blood vessel of the rats' tails of all the groups.

Osteocalcin, Osteonectin, crosslaps, Serum estradiol (E2) and progesterone were measured using ADVIA Centaur automated competitive chemiluminescence immunoassay (Bayer HealthCare).

Statistical analysis:

Comparison between different groups was carried out by one way analysis of variance (ANOVA) followed by Dunnett t (2-sided) multiple comparisons to detect significant differences among individual means of all groups. The level of significance was set at $p \leq 0.05$. Statistical analysis was generated using SPSS software for windows, version 16.0.

Results:

Effect of silymarin on serum estradiol and progesterone measured in OVX rats

Mean estradiol concentration on day 0 in unoperated group was 19.3±3.6, whereas estradiol concentration in control-operated was 36.4±16.4 on the same day. There was significant decrease in estradiol and progesterone concentration in all operated groups 30 days post operation. There was no significant change between day 30 and 60 pos-top in all operated groups. On the other hand there was also no significant change between different OVX groups in days 30 or 60 post-op in estradiol and progesterone concentration. Estradiol was significantly higher in normal group compare to other OVX groups in day 30 post operation. (Tables 1 and 2)

Effect of silymarin on serum osteocalcin in ovx rats:

The concentration of osteocalcin increased significantly in weeks 4 and 8 after surgery in all groups in which rats had been ovariectomized.The concentration of osteocalcin was not altered after oral administration of Silymarin, calcium or both in days 30 and 60 post operation compared with control- operated group which did not receive any treatment (table 3).There was no significant change in osteocalcin concentration between different treatment groups in certain days(30 and 60 post-op).

Effect of silymarin on serum osteonectin in ovx rats:

Osteonectin concentration increase significantly in control-operated which had not received silymarin or calcium treatment on day 30 and 60 post-op. Oral administration of silymarin or calcium or both from day 5 post operation did not induce any alternation in osteonectin concentration 30 and 60 days after surgery. Osteonectin concentration in those groups which did not receive any treatment up to week 4 post operation, showed significant increase primarily and then decreased significantly in day 60 post operation after oral administration silymarin, calcium or combination of both from day 30 post operation. There was a significant increase in osteonectin concentration in groups V, VI and VII, compare to other treatment groups and normal group in day 30 post operation (Table 4).There was no significant difference in osteonectin concentration between treatment groups on 60 post operation day.

Effect of silymarin on serum crosslaps in ovx rats:

Crosslaps concentration increase significantly in control-operated group which had not received silymarin or calcium treatment on day 30 and 60 post op. Crosslaps concentration in those groups which had received treatment from day 5 after surgery did not alter on day 30 and 60 post operation. Crosslaps concentration in those groups which had received treatment from week 4 post operation showed significant increase, primarily on day 30 post operation and then decreased significantly on day 60 post operation.(Table 5). There was a significant increase in Crosslaps concentration in groups V, VI and VII, compare to other treatment groups and normal group in day 30 post operation (Table 5).There was no significant difference in Crosslaps concentration between treatment groups on 60 post operation day.

Table 1: Estradiol serum concentration in different groups on days 0, 30 and 60 post operations

Normal	OVX VI	OVX V	OVX IV	OVX III	OVX II	OVX I	Control - operated	
19.3± 3.6	36.4±16.6	34.5 ±15.3	28.2±18.9	36.9±14.2	21.8±14.6	34.2±16.4	36.3 ± 15.7	Day 0
33.7± 8.3	*20.1±5.7	*17.3±3.8	*15.3±4.4	*17.9±4.9	*19.1±4.2	*18.8±6.2	*16.8±3.90	Day 30
20.6±4.3	17.8±7.1	15.7±6.7	13.4±4.2	15.2±4.6	16.8±5.2	16.5±3.9	15.6±4.3	Day 60

*= Significant difference between day 0 and other days ($p < 0.05$)

Table 2: Progesterone serum concentration in different groups on days 0, 30 and 60 post operations

Normal	OVX VI	OVX V	OVX IV	OVX III	OVX II	OVX I	Control - operated	
36/3± 1/2	35/3±/3	31/4±1/8	30/2±0/9	32/2±1/2	36/8±0/4	34/3±1/6	30/8±1/6	Day 0
19/8±/4	14/9±0/6	12/1±5/8	13/1±1/8	13/4±1/4	15/9±0/6	15/1±0/8	10/3±0/4	Day 30*
28/7±1/6	12/5±/3	13/3±1/2	13/9±/7	15/3±1/8	17/3±1/5	16/8±1/4	13/3±0/9	Day6 0**

*= Significant difference between day 0 and 30 post operation ($p < 0.05$). ** = Significant difference between day0 and day 60 ($p < 0.05$).

Table 3: Osteocalcin serum concentration in different groups on days 0, 30 and 60 post operation.

Normal	OVX VI	OVX V	OVX IV	OVX III	OVX II	OVX I	Control - operated	
76.6±36.6	74.6±28.6	73.9±36.4	78.6±29.7	74.5±26.6	77.8±22.2	75.6±19.5	76.6±25.6	Day 0
86.4±25.8	140.7±12.3	±14.2 148.2	135.5±13.3	145.2±9.8	136.7±14.8	±16.4 141.6	±10.6 139.5	Day 30*
110.6±26.6	142.3±8.4	±13.7 142.2	129.8±14.9	136.2±10.2	132.3±11.1	±15.8 140.6	±11.3 142.5	Day 60*

*= Significant difference between day 0 and other days ($p < 0.05$)

Table 4: Osteonectin serum concentration in different groups on days 0, 30 and 60 post operations.

Normal	OVX VI	OVX V	OVX IV	OVX III	OVX II	OVX I	Control - operated	
46.2±16.2	44.9±15.2	40.4±11.8	39.6±14.2	41.2±13.5	45.5±16.4	38.3±10.7	42.6±12.3	Day 0
49.9±12.2	±10.3 a*101.5	a*99.7±7.8	±6.4 a*104.6	45.8±7.6	64.3±9.4	53.9±7.6	*99.3±8.6	Day 30
47.3±11.4	**56.7±7.7	**61.7±10.2	±9.2 **69.1	51.7±8.2	62.7±8.6	50.7±6.4	*98.9±9.8	Day 60

*= Significant difference between day 0 and other days ($p < 0.05$). ** = Significant difference between day3 0 and day 60 ($p < 0.05$). a= Significant difference between OVX groups on day 30 post –op ($p < 0.05$).

Table 5: Crosslaps serum concentration in different groups in days 0, 30 and 60 post operations.

Normal	OVX VI	OVX V	OVX IV	OVX III	OVX II	OVX I	Control - operated	
516.8±87.3	621.6±201.3	±127.5 648.8	±179.3 657.9	±163.7 587.4	±183.2 633.6	±123.5 640.7	636.6±177.6	Day 0
±102.3 547.8	±247.8 a*1050.7	±231.7 a*1010.2	±221.7 a*1037.7	±215.6 589.3	±218.8 720.0	±248.3 654.1	±202.3 a*989.8	Day 30
±142.7 625.7	±217.7 **627.2	±182.8 **692.3	±297.3 **715.3	±205.7 665.2	±210.0 698.7	±211.4 597.9	a887.7±186.6	Day 60

*= Significant difference between day 0 and other days ($p < 0.05$). ** = Significant difference between day30 and day 60 ($p < 0.05$). a= Significant difference between OVX groups on day 30 post –op ($p < 0.05$).

Discussion:

The objective of this study was to examine the effect of Silymarin alone and in combination with Calcium on bone formation, resorption and turnover in *ovariohysterectomized* rat model. Bone markers are useful tools for the management of bone diseases, such as osteoporosis. The most outstanding example of this situation is Postmenopausal osteoporosis in which estrogen-deficient state is characterized by bone fragility, as the balance between bone-resorption and bone-formation shifts towards increased levels of bone-resorption [19]. In our study the same situation after ovariohysterectomy was expected. Mean estradiol concentration on day 0 in unoperated group was 19.3±3.6, whereas estradiol concentration in control-operated was 36.4±16.4 on the same day. This difference may be due to estrous cycle as we can see approximately the same figure in group OVX II.

Significant decrease in stradiol and progesterone levels on the 30th day was observed compared to day 0 after ovariohysterectomy in all operated groups. Administration of oral silymarin alone and in combination with calcium could not alter the decreased estradiol and progesterone rates. Estrogenic effects of oral silymarin via EBR receptors were well documented by Kummer *et al* [12].In our study ovaries were eliminated from the body as a source of estradiol and progesterone production. Presence of Silymarin in the body system cannot increase the level of estradiol and progesterone. On the other hand, the ovaries, by affecting estradiol receptors can only dramatize estradiol effects in the body of rats. Regarding extraction of ovaries as a source of hormone production, decrease in the level of estradiol and progesterone hormones seems logical.

Serum concentration of osteocalcin was measured on days 30 and 60 after ovariohysterectomy and showed elevation in concentration in week 4, reaching its maximum concentration in week 8 after surgery. This elevation was the same in all groups. Many studies have been done in humans and animals, which confirm the increase of bone turnover after ovariohysterectomy and ovariectomy; Peris *et al* [20] determined biochemical markers of bone metabolism after surgical menopause in humans. These markers included formation marker osteocalcin. Three months after the operation all formation and resorption markers determined in this study increased. Itoh *et al*. [21] did a similar study with mature cynomolgus monkeys. These were ovariectomised and evaluated over a period of 16 months. Serum osteocalcin values increase 168% compared to sham operated animals. Newton *et al* [22] determined the effects of ovariectomy on the trabeculae of ovine iliac bone. Blood samples were drawn at ovariectomy and in three month intervals up until twelve months. Osteocalcin concentrations were significantly higher in the OVX groups compared to the controls. Lukacs *et al*. [23] demonstrated that women in early menopause years (40-52 years) had elevated osteocalcin values. In the present study increase in osteocalcin level four weeks after surgery revealed that osteoblasts have became more active compared to presurgery period due to an imbalance in bone turnover, so the secretion of osteocalcin was increased after surgery. Rucinski *et al* [24] investigated the effects of estradiol and some resveratrol like

silymarin on osteocalcin gene expression in Osteoblasts primary cells and found that despite estradiol, Silymarin could not alter osteocalcin gene expression in osteoblast primary cells. Nagla *et al*. [25] compared antiosteoporotic and selective estrogen receptor modulator activity of silymarin with ethinylestradiol in ovariectomized rats, and revealed that despite ethinylestradiol, silymarin could not increase the level of serum osteocalcin in ovariectomized rats. In our study the concentration of serum osteocalcin in all groups, especially in those that received silymarin alone or in combination with calcium did not decrease and silymarin could not decrease serum osteocalcin concentration. Silymarin simulate its estrogenic effects via estrogen β receptors whereas osteoblasts, which are responsible for osteocalcin secretion contain estrogenic α receptors, and perhaps this is the reason why silymarin is not able to affect osteoblasts and did not alter serum osteocalcin concentration. Many of the effects of estrogen in the bone are mediated via IGF1 and TGFb1.TGFb1 is also a proliferative factor involved in proliferation of osteoblasts and therefore an antiosteoporotic growth factor [26]. Silymarin probably stimulate osteoblasts activity and osteocalcin secretion via bone growth proliferative factors but not via estrogenic inhibitory receptors, therefore silymarin cannot reduce osteoblasts activity and osteocalcin production via osteoblast estrogenic receptors. In addition, there was no significant difference between OVX groups in days 30 or 60; and it demonstrates that neither silymarin nor calcium could alter osteocalcin concentration and their combination were also ineffective against osteocalcin surge.

As seen in the results in the group which did not receive any treatment and those that received treatment from week 4 after surgery, crosslaps and osteonectin concentration were increased significantly on days 30 and 60 postoperation. Gaumet *et al* [27] studied the influence of ovariectomy on bone metabolism in very old rats and found an increase in bone resorption markers pyridinoline and deoxypyridinoline seven weeks after OVX compared to the controls. The absence of estrogen caused an increase in osteoclast activity, post operation. This increase in osteoclast activity when there was lack of estrogen was portrayed by the increase in crosslaps and osteonectin activity after operation. In the present study increase in osteoclast activity was associated with elevation of crosslaps and osteonectin concentration, bone turnover and osteoblasts activity (osteocalcin concentration was also increased in weeks 4 and 8 after operation).From all of the above it is concluded that, after surgery in groups that did not receive treatment, bone turnover has occurred. On the other hand, concentration of crosslaps and osteonectin in those groups that received silymarin or calcium or both after operation did not increase and the elevated level of osteonectin and crosslaps in those groups that had received treatment after 4 weeks decreased significantly 4 weeks later on day 60 after operation; and it reflects the inhibitory effect of calcium, silymarin or mixture of both on osteonectin and crosslaps elevation. There was no significant difference between OVX groups I, II and III in days 30 and 60. There was also no significant difference between those groups that received treatment after day 30 post-op (OVX groups IV, V and VI); and it means Silymarin, calcium and the combimation of both drugs had the same effect on osteonectin and crosslaps in different groups in a certain day.

Bone resorption and intestinal calcium absorption are regulated by calcitriol (vitamine D hormone) and parathyroid hormone. An increase in serum calcium concentrations would result in a decreased production of these two hormones and an increase in calcitonin. Calcitonin is the antagonist of parathyroid hormone in the bone, which means that it can occupy the paratormone receptors in the bone. Greenberg *et al* [28] found that estrogen stimulated PTH secretion by rapid, direct, and specific effects on parathyroid cells and hence, estrogen may, therefore, be important in calcium homeostasis via their direct stimulatory effect on PTH secretion. This study showed that serum crosslaps had a tendency towards lower concentrations in the groups of animals that were fed with higher calcium diet per day. Mosekilde *et al* [29] show that calcium restriction in diets can cause bone loss. Our results also confirm these studies and show that concentration of crosslaps and osteonectin in those groups that had received calcium in their diet did not increase and the elevated level of osteonectin and crosslaps in those groups that had received treatment after 4 weeks decreased significantly 4 weeks later on day 60 after operation. The high rate of calcium in serum results in a decrease in paratormone secretion. Decline in paratormone secretion can cause decrease in the number and activity of osteoclasts and consequently a decrease in bone resorption. Jung Ha Kim *et al* [30] showed that Silibinin inhabits osteoclast differentiation mediated by TNF family members. Silibinin is a potential therapeutic agent that encumbers osteoclastic bone resorption [31]. In our study silymarin was also shown to have protective effect against bone resorption as the concentration of osteonectin declined in those groups that had received silymarin.

In summary, the current report demonstrated that calcium and silymarin alone or in combination with each other have a preventative effect against osteoporosis and this treatment may dictate its effect via bone resorption processes, and in each of the treatments, silymarin or calcium has its own independent way to compromise osteoclastic bone resorption activity. None of the treatments showed priority compared to others regarding their effect on selected parameters. Incidentally, it is necessary to investigate the effects of silymarin and calcium on other bone resoption, absorption and turnover markers in the future.

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