Experimental ovarian transplantation
on stomach for bone repair in ovariohysterectomized rabbits[1]

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ABSTRACT

PURPOSE: To evaluate the bone repair process in ovariohysterectomized rabbit submitted to an ovarian transplant to stomach that may supplying some quantity of estrogen occurs to improve bone healing.

METHODS: In 20 female rabbits three holes of 1, 2 and 3mm diameter in tibial shaft were made and after that all animals received OHE through a ventral incision and they were randomly divided into two groups of ten rabbits each. In group one, animals received one of their self-ovaries that transplanted on serosal layer of stomach and group two did not receive treatment. Animals were kept during bone healing for a period of 45 days and radiological, biochemical, biomechanical and histopathological evaluation.

RESULTS: The tibial defects in group one healed completely after 45 days and had more callous than second group. There is significant difference between two groups after operation in 21, 28 and 35 days about estrogen, progesterone and phosphatase Alkaline. The maximum forces in group one, were significantly higher than that for the group two.

CONCLUSION: Ovarian transplantation prevents the effects of ovariohysterectomized on bone healing of rabbit tibia, suggesting that unilateral transplanted ovaries can substitute for the action of ovaries on the skeleton in ovariohysterectomized animals.

Key words: Hysterectomy. Ovary. Organ Transplantation. Rabbits.

Introduction

Considering that the removal of the ovaries can prevent complications such as breast tumors, and also asked the owner's to prevent pregnancy and oestrus animals comes[1], the ovary is the major source of estrogen that is involved in the bone remodeling process, being responsible for the balance between resorption and bone formation[2].

The effects of estrogen deficiency lead to reduced bone matrix, osteoblastic activity and loss of calcium and phosphate deposition in bone[3,4]. Hormone replacement therapy can prevent unintended bone healing delay; but the drugs have certain side effects with long-term use[5] such as increasing risk of gallbladder disease and breast cancer[6].

After ovariectomy, the absorption of bone prevails over osteogenesis and the fracture healing is poor in quality[7,8]. This might reflect a failure to reproduce certain aspects of gonadal function. Auto transplantation of an ovary to stomach wall, which drained exclusively by the portal vein may prevents this complication. Circulating estradiol levels are inadequate to initiate estrus but may be sufficient to prevent the effects of estrogen deficiency after OHE.

The aim of this study was to evaluate the bone repair process in ovariohysterectomized rabbit submitted to an ovarian transplant to stomach that may supplying some quantity of estrogen occurs to improve bone healing.

Methods

The experimental protocol used was reviewed and approved by Kahnooj Institutional Animal Care and Use Committee; 20 female rabbits of New Zealand white strain were used in the present study. All animals had free access to food and water and were individually housed in a 12 hours light-dark cycle.

All animals were anesthetized by intramuscular injection of xylazine (5 mg/Kg of body weight) and ketamine hydrochloride (30 mg/Kg of body weight) and under sterile conditions 5 cm incision was made on the proximal-anterior part of right tibia. The periosteum was elevated with a periosteal elevator and retained by a selfretaining retractor then three holes of 1, 2 and 3mm diameter in tibial shaft were made with a hand drill. The holes pass through the both cortex. Physiological saline solution was used during drilling to prevent overheating. After that all animals received OHE through a ventral incision and they were randomly divided into two groups of ten rabbits each.

In group I; animals received one of their self-ovaries that transplanted on serosal layer of stomach body without vascular anastomoses[9]. Group II did not receive treatment.

Antibiotic prophylaxis with cefonicid sodium (100 mg/kg) was administered before and during the 4 days following surgery to minimize any complications. For pain prophylaxis during the initial 48 h postoperatively four injections of carprofen (4 mg/kg) were administrated.

Animals were kept during bone healing for a period of 45 days. Process of holes filling with callus formation was evaluated on radiographs taken on days zero, 15 and 30 and 45. Radiographs in the lateral views were taken of all tibias in the Faxitron Cabinet X-ray System (43855A, Hewlett-Packard, IL, USA). A high-resolution film and 40 KV/6 min radiation was used. The description and evaluation of the fracture healing was performed in a blinded manner for all the test groups.

Radiographic Evaluation was scored as follows: 0, no callus formation; 1, filling 1mm holes; 2, filling 2mm holes; 3, filling 3mm holes with callus formation.

Blood samples were taken from the marginal veil of rabbits. Estrogen, progesterone, and alkaline phosphatase were analyzed on days zero (before surgery), seven, 14, 21, 28, 35 and 45 days.

The rabbits were euthanized at 45 days post operation; each tibia was placed on the breaking test. Before testing, the specimens were thawed and all musculature was carefully removed so that the bony structures were not damaged. The tibias were continuously moistened with isotonic saline solution. For biomechanical analysis we used the bending test machine (7100L, Gotech, Thaiwan). Maximum load is a widely used parameter for mechanical evaluation, and represents the maximum force that a material can sustain before failure[10].

The measuring range was from 2 to 200 Newton (N) at a relative accuracy of 0.2% at 0.4% nominal force. A primary force of 1N to fix the tibia on the device was chosen. The software (Test Xpert, Gotech, Thaiwan) continuously recorded the force. The investigation was manually stopped by the first linear displacement visibly to prevent damage or fracture of the callus formation. The software program indicated the maximum load. This procedure was performed in a blinded manner with regard to the test groups.

For histopathological analysis, all specimens were fixed with 10% neutral buffered formaldehyde (pH 7.2) for 72 h and decalcified with 15% neural EDTA for three weeks. Each bone defect area was sagittally sectioned perpendicular to the defect and dehydrated in a series of concentrations of alcohol (80% up

to absolute). The samples were embedded in paraffin and sectioned at 5 μm thickness. The sections were stained with hematoxylin and eosin. Assessment was performed by a blinded assessor according Table 1.

TABLE 1 - Histologic scoring scale.

Parameters	Scores	3	2	1	0
Osseous formation		≥75%	50%≤ - ≤75%	25%≤ - ≤50%	25% ≥ -
Metachromasia of bone matrix		Normal	Slightly reduced	Predominantly reduced	No metachromasia
Connective tissue presentation		25% ≥	25%≤ - ≤50%	50%≤ - ≤75%	≥75%
Trabecular thickness		≥60μ	20μ≤ - ≤60μ	20μ ≥	0
Inflammatory reaction			Sever reaction	Moderate reaction	No reaction
Deformity formation				No Deformity	Deformity

Values in tables are reported as mean ± SD. Statistical analyses were performed using the Mann-Whitney U-test, and SPSS for Windows version 11.0 software program; $p<0.05$ was considered significant.

Results

No sign of inflammation was seen in all animals.

The tibial defects of all ten rabbits in group I healed completely after 45 days.

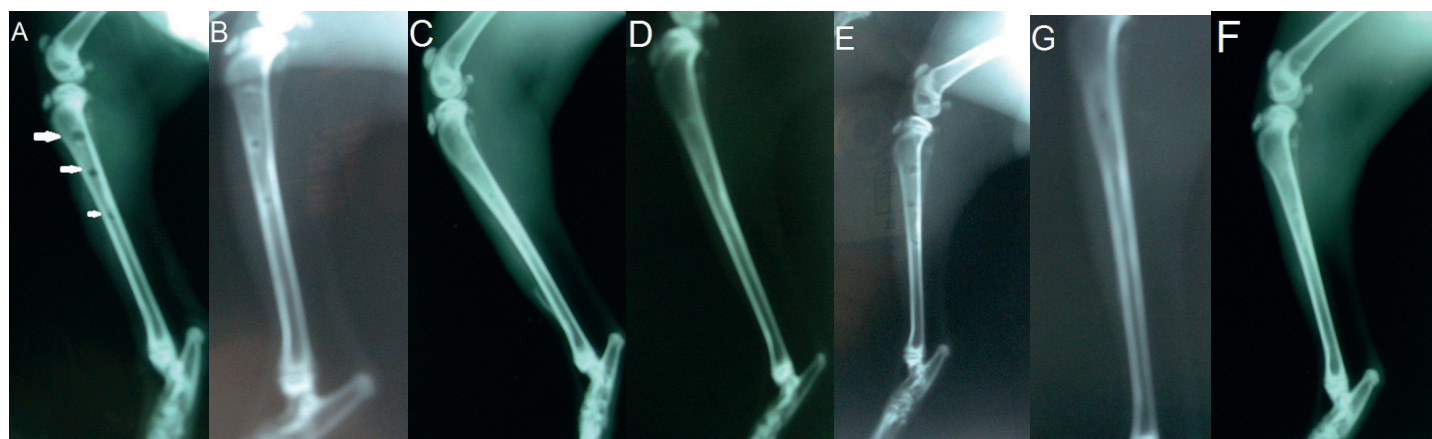In relation to constitute callous in both groups, there

FIGURE 1 - X-rays of the experimental defects in tibial shaft during the healing period of 45 days. The arrows indicate the three holes of 1, 2 and 3mm

diameter immediately after the operating procedure (**A**). Radiographs showing the process of holes filling with callus formation in group I after 15 (**B**), 30 (**C**), and 45 days (**D**), and in group II after 15 (**E**), 30 days (**F**), and 45 days (**G**).

TABLE 2 - Summary of the mean radiological grades. Values are given as mean grade ± standard deviation.

Group	Days			
	0	15	30	45
I	0 ± 0	0.040 ± 0.55	2.80 ± 0.44*	3.00*
II	0 ± 0	0.20 ± 0.44	1.00 ± 0.70	1.80 ± 0.83

*There are meaningful variations in 30 and 45 days and group one had more callous formation than group two, p<0.05.

There is significant difference between two groups after operation in 21, 28 and 35 days about estrogen and in 21th day to the end of the research period about progesterone. About phosphatase Alkaline, there are meaningful differences between two groups in 14, 21, 28 and 35 days (Table 3).

TABLE 3 - Mean values and standard deviations, of estrogen, progestron and alkaline phosphatase levels in two groups on different days.

Group (Parameter)	Days						
	0	7	14	21	28	35	45
I (estrogen)	117.20 ± 26.28	119.80 ± 23.86	119.80 ± 17.28	113.40 ± 14.51*	108.20 ± 18.09*	104.20 ± 11.45*	101.20 ± 23.16
II (estrogen)	116.40 ± 24.76	117.60 ± 13.37	116.40 ± 8.73	88.80 ± 12.03	81 ± 7.71	82 ± 3.08	80.20 ± 11.08
I (progestron)	0.96 ± 0.26	0.20 ± 0.30	0.87 ± 0.17	0.67 ± 0.18**	0.65 ± 1.21**	0.76 ± 0.18**	0.57 ± 0.23**
II (progestron)	0.34 ± 0.37	0.15 ± 0.23	0.01 ± 0.24	0.97 ± 0.13	0.96 ± 0.06	0.14 ± 0.25	0.01 ± 0.19
I (alkaline phosphatase)	99.88± 64.52	219.80 ± 39.40	227.20 ± 46.63***	204.80 ± 41.10***	188.20 ± 27.27***	182.60 ± 36.63***	152.60 ± 30.44
II (alkaline phosphatase)	120.60 ± 29.72	165.20 ± 65.21	146.80 ± 32.80	150 ± 23.10	133.20 ± 22.80	125.80 ± 24.03	105.60 ± 36.23

*There is significant difference between two groups after operation in 21, 28 and 35 days, p<0.05.
**There is significant difference between two groups on 21th day to the end of the research, p<0.05.
***There are meaningful differences between two groups in 14, 21, 28 and 35 days, p<0.05.

To prevent damage of the callus formation, the maximum forces were calculated when assessor can see the first linear displacement of samples. The maximum forces in group I (182.80 ± 15.38 N) were significantly higher than that for the group II (134 ± 14.85 N) (Table 4).

TABLE 4 - Maximum forces ± standard deviations (in Newtons) to see the first linear displacement of samples.

Group	Maximum force
I	182.80 ± 15.38*
II	134 ± 14.85

*The maximum forces in group one was significantly higher than that for the group two.

Pathological evaluations showed that the osseous trabecula became thinner and approximately disrupted in group II, whereas in group I it became massive, thicker and closer to normal (Figure 2A) and haversian systems had been formatted in new bone (Figure 2B). In group II the outer cortex had not been formatted completely (Figure 3A) and connective tissue was observed in the outer cortex and the bone healing parameters were significantly lower than group I (Table 5).
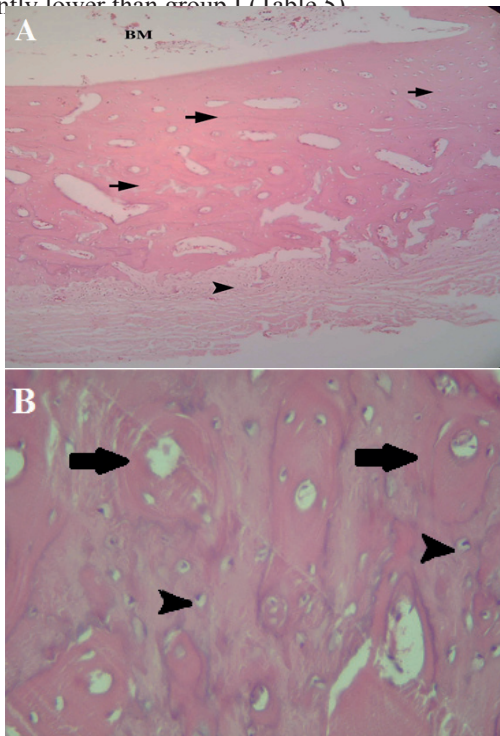

FIGURE 2 - Pathological evaluation of group one. **A)** The inner cortex has acceptable osseous formation (arrows) and in outer cortex there are some connective tissue and callus (arrowhead), (H&E, ×16). **B)** Haversian

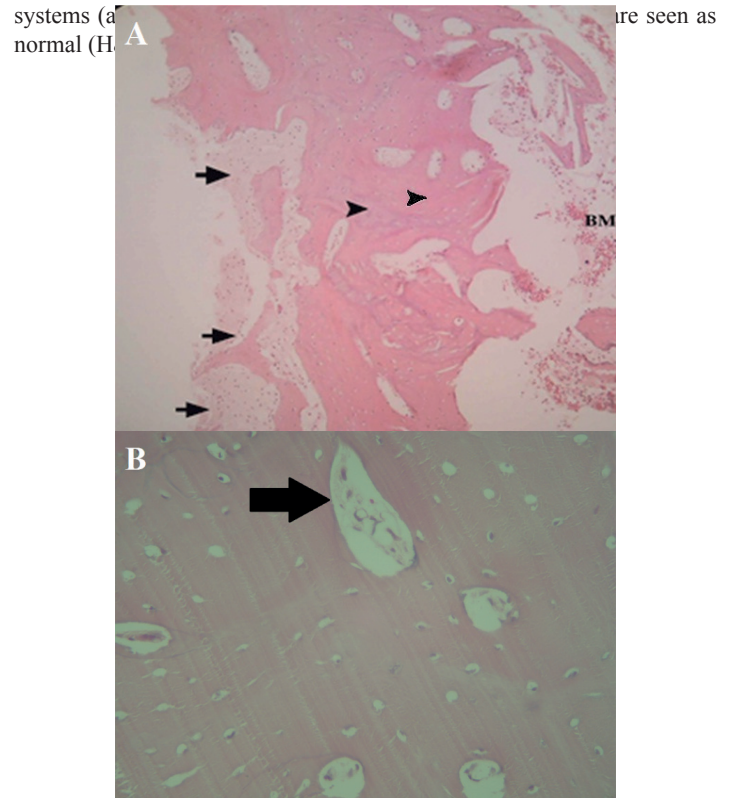systems (a... ...re seen as normal (H...

FIGURE 3 - Pathological evaluation of group two. **A)** Osseous formation in the inner cortex was incomplete (arrowhead) and connective tissue was observed in the outer cortex (arrows) (H&E, ×16). **B)** In the newly formed bone Lacunas is seen wider than normal, which can be indicative of osteoporosis (arrowhead) (H&E, ×64). BM=Bone Marrow.

TABLE 5 - Results of the histologic grading scale.

Characteristics (maximum scores) Group	Deformity formation (2)	Inflammatory reaction (2)	Connective tissue present (3)	Metachromasia of bone matrix (3)	Trabecular thickness (3)	Osseous formation (3)	Total scores (16)
One (n=10)	1.00 ± 0.0	2.00 ± 0.0	3.00 ± 0.0	3.00 ± 0.0	2.60 ± 0.55	2.60 ± 0.55	14.2 ± 1.1*
two (n=10)	0 ± 0	2.00 ± 0.0	2.00 ± 0.0	2.0 ± 0.0	2.20 ± 0.55	2.20 ± 0.44	10.4 ± 0.99

*Group one is significantly different from group two, $p < 0.05$.

In the newly formed bone Lacunas is seen wider than normal, which can be indicative of osteoporosis (Figure 3B). Transplanted ovary and stomach both seem healthy and no inflammatory reaction was seen.

Discussion

Ovariohysterectomy is the safest and most effective treatment for pyometra[11,12] and it is a radical treatment for postoestrus metritis when it recurs following failure of medical treatment and also that is, sterilisation, as many owners complain of the manifestations of heat with vulvular discharge, as well as the problems associated with repeated mattings[13] and finally, ovariohysterectomy is a tool used for combating pet overpopulation[14].

The bone remodeling function is always in balance and depends on systemic and local factors. Amongst systemic factors, estrogen is probably the most important hormone responsible for the maintenance of normal bone turnover[15], after elimination of ovary, the absorption of bone prevails over osteogenesis and bone loss occurs as soon as 1 month after OHE[7].

Xu et al.[16] reported a reduction in callus and bone mineral density in femoral fracture healing after Ovariectomy in rats, also osteoporotic changes occur and healing of fractures was poor quality. Similarly in this study in group II after 45 days in newly formed bone the lacunas were much wider than normal which could be indicative of osteoporosis.

In a study ovariectomized rabbits were examined with a combination of estradiol and progesterone for a period of 42 days. Results indicated that estradiol and progesterone prevent bone loss by either inhibited reducing the number of osteotrabecula and bone callus[18].

In research transplanted allogeneic ovaries secreted estrogen at normal levels. Furthermore, bone loss was prevented to a certain extent[19]. In our study, group II showed significantly reduction of estrogen whereas in group I this reduction was very low, especially on 21 to 35 days after transplantation. At the end of 45 days, estrogen was less than normal, as the day zero, but it was significantly higher to compare the group II.

Tobias et al.[20] investigated whether renal capsular or subcutaneous ovarian transplants prevent the effects of ovariectomy on histomorphometric indices of tibiae and concluded that ovarian transplantation largely prevents the effects of ovariectomy such as reduction in cancellous bone volume. We investigated unilateral ovarian auto transplantation on serosal layer of stomach to prevent the effects of ovarian hormones elimination on bone healing after OHE. Circulating estradiol levels after transplantation of the ovary to the portal vein drainage area, which is partially metabolized by the liver, are inadequate to initiate estrus but may be sufficient to supplying some quantity of estrogen to improve bone healing on ovariohysterectomized animals. Our results showed that ovarian transplantation led to a better remodeling of regenerated bone as measured by radiologic, histologic and biomechanical analysis and the process of bone repair in the ovarian transplanted group was relatively better than the control group.

Compression testing is recommended for testing the mechanical properties of bone[21]. Qiao et al.[7] demonstrated that on 50 days after ovariectomy in rats, tibia fracture healing and biomechanical strength is 24% and 22% lesser than normal rats. We used load presser on tibia and biomechanics results showed that the strength of specimens in group I is higher than the group II, so ovarian transplantation could be effective to bone strength.

The transplanted ovary tissues under the renal capsules of mice were accepted without using immunosuppressants[19], similarly in the present study any signs of rejection or inflammatory was not seen.

Gallagher et al.[17] compared the effects of administering estradiol to osteopenic ovariectomized rats with those of ovarian transplantation. Both groups largely restored indices of estrogenic exposure. Animals receiving ovarian transplants also showed a small increase in serum progesterone.

In this study progesterone were significantly different between two groups on 21 to 45 days.

Alkaline phosphatase had increased in both groups after day zero, which would be specifically due to bone damage, but it was significantly different between groups on 14 to 35 days, this reason was not clear.

Conclusion

The ovarian transplantation prevents the effects of ovariohysterectomized on bone healing of rabbit tibia, suggesting that unilateral transplanted ovaries can substitute for the action of ovaries on the skeleton in OHE animals.

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