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探討薯蕷苷對停經大鼠之神經免疫功能的影響：一併探討細胞層面之效果

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Abstract: Objective: The aim of this work was to determine the effects of oral administration of dioscorea on the morphometric and mechanical properties of the femur in ovariectomized (OVX) rats.
Design: Female Wistar rats that had undergone surgery for ovariectomy were used as a model of menopause and osteoporosis. Four weeks after surgery, the animals were given oral dioscorea (0, 250, 750, or 1500 mg/kg/day) for 27 days, then the porosity, mineral fraction, stiffness, and toughness of the femur and the ultimate force needed to break the femur were measured.
Results: Ovariectomy resulted in an increase in porosity, but a decrease in the mineral fraction. Subsequent chronic administration of dioscorea reversed the effect on porosity and increased the ultimate force of the femur in OVX rats, but did not affect the bone properties of sham-operated rats.
Conclusion: Chronic administration of dioscorea may inhibit bone loss and enhance bone strength. This study provides insight into the role of dioscorea in bone remodeling and osteoporosis during the menopause.

Effects of Dioscorea on the Morphometric and Mechanical Properties of Bone in Ovariectomized Rats

Running title: Effects of Dioscorea on the Bone

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ABSTRACT

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INTRODUCTION

Osteoporosis is a highly prevalent disease in postmenopausal women. The bone loss is caused by the imbalance between bone formation and resorption, which increases the risk of fracture.[1] Fifty percent of Western females and 33% of males are reported to suffer from osteoporosis.[2] Decreased blood levels of sex hormones are thought to be involved in osteoporosis, as postmenopausal syndrome is significantly improved by hormone replacement therapy, especially a combined estrogen-progesterone regimen.[3] Thus, administration of foods containing hormone or hormone precursor might be effective in preventing osteoporosis.

Dioscorea (wild yam) has long been used as a Chinese medicine for improving gastrointestinal, sensory, memory, and sexual-related functions and hot flush and frequency of urination in postmenopausal women. Animal studies have been used to evaluate the effect of dioscorea on osteoporosis,[4] diabetes,[5] and hyperlipidemia,[6] symptoms which are very common in postmenopausal women. Furthermore, effects of dioscorea on bone function at the cellular and genomic levels have been reported.[4,7] Diosgenin, the main steroidal saponin in dioscorea.[8,9] is used to manufacture steroidal hormones, such as progesterone, estrogen, testosterone, and cortisone,[10,11] by in vitro chemical modification.[12] Because diosgenin stimulates the growth of the mammary epithelium[13] and inhibits weight gain following ovariectomy,[14] it has been suggested to be responsible for the effects of dioscorea.

The mechanical properties of bone are determined by its morphometric properties, e.g. its mineral content[15,16,17] and porosity.[18] Hernandez *et al.* found that the volume fraction of bone (1 minus the porosity) and the mineral fraction (bone mineral content divided by the dry weight of bone) are not correlated, suggesting that they are two independent parameters.[19] The bone mineral fraction and bone volume fraction may be affected differently during bone remodeling. In addition, the bone mineral fraction is considered as “bone quality”, while the

bone volume fraction is used as an index of "bone quantity". Analyzing the relationship between the morphometric and mechanical properties of the bone of OVX animals may provide insights into the pathophysiological role of osteoporosis in menopausal animals.

OVX rats are used as a menopausal animal model because the changes in biochemical and physiological function seen in these animals are similar to those in menopausal women,[20] i.e., decreased levels of progesterone and estrogen,[21] an increased risk of cardiovascular disease,[22] and an enhanced rate of bone loss,[7,23] as well as increased anxiety levels.[24] As far as we are aware, there are no published studies on the effect of dioscorea on the morphometric and mechanical properties of bone in OVX rats. The aim of this study was to use dual energy X-ray absorption (DEXA) scans and a three-point bending test to evaluate the effects of chronic oral administration of dioscorea on the morphometric and mechanical properties of the femur in OVX rats that had undergone ovariectomy four weeks previously.

METHODS

Animals

Adult female Wistar rats (258.8 ± 3.94 g; n=90; National Laboratory Animal Center, ROC) were used and housed in groups of five in acrylic cages (35 × 56 × 19 cm) in an animal room with a 12 h light-dark cycle (lights on at 07:00 hr) with food and water provided *ad libitum*. Each animal was handled for 15 min per day on 2 consecutive days prior to the experiment. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Chung Shan Medical University.

Surgery and general procedures

The rats were randomly divided into two groups which either underwent surgery for ovariectomy (OVX group; n=60) or were sham-operated (n=30), as described in our previous report.[25] After surgery, the rats were housed individually in plastic cages (25 × 41 × 19 cm) for about 10 days for recovery, then re-grouped in their home cages.

Administration of dioscorea

Dioscorea (D. L. alata. Var. purpurea (Roxb.) M. Pouch; Tainung No. 1 Shan-Yao) was purchased from Ming-Jean town, Nan Tao County, Taiwan. The yam tubers were cleaned, peeled, sliced into 1 cm wide slices, and boiled for 30 min to inhibit the browning reaction. The cooked sample was then placed in a grinder set at a moisture level of around 10% and was milled to a flour that passed through a 60 mesh sieve which was stored at -25□ until use. The dose of dioscorea was freshly prepared before use by adding double distilled water to the flour and mixing. From day 28 after ovariectomy or sham operation, the animals were given dioscorea (250, 750, or 1500 mg/kg/day) or vehicle (distilled water) by oral gavage for 27 days. Similar conditions of 4-6 weeks after ovariectomy[14,26,27] and a duration of treatment for osteoporosis of 4-6 weeks[27,28,29] have been used by other authors using the rat menopausal model. The dosage used in the present study was based on the results of our previous study in which treatment with 250-1500 mg/kg/day of dioscorea for 27 days caused pronounced changes in behavior and biochemistry[25] and our wish that the amount of dioscorea administered per day be less than 10% of the average food intake (around 10 g) to avoid an effect on calorie intake; in the highest dose rats, the dose was about 450 mg, i.e. 4.5%. The rats were then sacrificed using CO_2 and their femora immediately removed.

Preparation of femora and determination of the morphometric and mechanical properties

During the removal of the muscle and the fibrous periosteum, the femora were kept wet

in distilled water. After defatting in chloroform[30] and drying, the right femur was used to sequentially measure the morphometric parameters of wet weight, total volume, dry weight, and mineral content to calculate the porosity and bone mineral fraction. The left femur was kept at −20□ till mechanical testing. A previous report has shown that drying, re-wetting, and freezing have no effect on the mechanical properties of bone.[31]

Measurement of the wet weight: The right femur was placed in an unstoppered glass vial containing distilled water and the vial placed in a vacuum desiccator for 90 min to remove air diffusing out of the bone.[32] After gently wiping off the water on the surface of the specimen, the femur were then weighed to give the wet weight (W_W) using an analytical balance (AE240-S, Mettler, Taiwan).

Measurement of the total volume: According to the theory of porous media,[33] the porous structure of the bone consists of the 'solid skeleton' (bone frame volume; V_B) and the 'interstitial fluid', (void volume; V_P).[34] Instead of using a conventional caliper or mathematical equations, we have, by combining Newton's third law and Archimedes' principle, developed a novel and accurate method to directly measure the total volume (V_T; $V_T = V_B + V_P$; cm^3) of the femur.[18,35] Briefly, each right femur was suspended by a thin silk yarn and fully immersed in water in a beaker on the analytical balance and the buoyant force ($\vec{B}$; gw) read directly from the balance display . The total volume, V_T, of the femur was calculated as V_T = buoyant force/0.9971, where 0.9971 is the density (g/cm^3) of distilled water at 25 °C and 1 atm.[36]

Measurement of the dry weight: After measuring the total volume, each right femur was placed in an incubator at 50 °C for 72 h to remove the interstitial fluid until a constant weight (< 0.05% change) was obtained in weighings at 1 h intervals. The dry weight (W_D) was immediately measured on the analytical balance.

Measurement of the bone mineral content: To measure the bone mineral content (W_M),[37] each specimen was immersed in 10 cm of distilled water,[38] which has been demonstrated to simulate the surrounding soft tissue in the *in vivo* situation,[39] and underwent DEXA scans in the EXPERT-XL system (Lunar, Madison, WI, USA). The DEXA scan is a common technique for assessing bone mineral density.[40] Our previous study showed that the measurement of the bone mineral content is constant irrespective of the scan direction.[18] Each specimen underwent three scan trials with an inter-trial-interval of one week which were performed by a senior radiologist with more than five years' experience. The code of the femur was reassigned before each trial so as to perform a single-blind test.

Calculation of the morphometric parameters: The values of two morphometric parameters, bone porosity and bone mineral fraction, were calculated as follows. In the saturated right femur, the void space is filled with W_f g of distilled water. The void volume, V_P, was calculated using the equations $V_P=W_f/0.9971$ and $W_f=W_W-W_D$. The porosity, P, was calculated using the equation $P=V_P/V_T$. The bone mineral fraction was calculated by dividing the bone mineral content, W_M, by the bone dry weight, W_D.

Evaluation of the mechanical properties: Before mechanical testing, the left femur was soaked in saline at room temperature for 12 h. A material testing system, INSTRON 4464-Standard (Instron, USA), was used to perform the "three-point bending test" (Fig. 1A). The rate of compression was 0.5 mm/min and the relationship between load (force; N) and displacement (mm) was recorded at a sampling rate of 10 Hz. The slope of the linear section of the load-displacement curve gives the stiffness (S; N/mm). The force required to fracture the bone is the ultimate force (F_{ult}; N), which causes the ultimate displacement (D_{ult}; mm). The work (energy) need to fracture the bone, or the toughness (N□mm), was calculated as the area under the curve from zero displacement to ultimate displacement (Fig. 1B).

Data analysis

To analyze the effects of surgery and dioscorea treatment, two-way analysis of variance (ANOVA) was carried out. When interactions existed, the independent *t*-test was used to analyze the effects of ovariectomy. To evaluate the effects of dioscorea treatment, one-way ANOVA was performed, followed by the least-significant difference (LSD) post hoc test. All results are expressed as the mean ± SEM. The level of significance was defined as $P < 0.05$.

RESULTS

At four weeks after ovariectomy, the percentage change in the body weight in the OVX rats was significantly higher than that in sham-operated rats (df=88, t=-2.377, $P = 0.02$; Fig. 2A). A subsequent four weeks of dioscorea treatment did not affect the change in body weight over this 4-week period in either OVX or sham-operated rats (Fig. 2B). Two-way ANOVA of surgery by dose was used to analyze the data. In addition to the main effects of surgery on stiffness ($F(1,83)=13.848$, $P < 0.001$), ultimate force ($F(1, 81) = 27.359$, $P < 0.001$), and toughness ($F(1, 83) = 21.174$, $P < 0.001$), there were interactions between surgery and dose for porosity ($F(2,83) = 3.676$, $P = 0.030$) and ultimate force ($F(2,81) = 3.306$, $P = 0.042$). An independent *t*-test revealed that, at 4 weeks after surgery, the porosity of the femora was higher (df = 20, t = -2.395, $P = 0.027$), but the mineral fraction (df = 20, t = 2.629, $P = 0.016$) lower, in OVX rats than in sham-operated rats. ANOVA followed by the LSD test showed significant differences between the OVX rat groups in porosity ($F(3,56)=3.264$, $P = 0.028$) and ultimate force ($F(3,54)=3.158$, $P = 0.032$) of the femora. As shown in Table 1, the post hoc test indicated that, in OVX rats, oral administration of dioscorea at the dosage of 750 or 1500 mg/kg for 27 days returned the porosity of the femora to control levels (both P values < 0.05); furthermore, all three dosages of dioscorea increased the ultimate force (all P values <

0.05). In contrast, dioscorea treatment did not affect the morphometric and mechanical properties of the femur in sham-operated rats. There were no effects on bon mineral fraction, stiffness, or toughness.

DISCUSSION

Four weeks after ovariectomy, the body weight increase in OVX rats was significantly higher than that in sham-operated rats, consistent with previous reports for menopausal animal models.[14,26,27] Ovariectomy also resulted in an increase in porosity and a decrease in bone mineral fraction in the femora. Subsequent chronic administration of dioscorea reversed the effect on porosity and increased the ultimate force of the femora in OVX rats, but did not affect the bone properties of sham-operated rats.

OVX rats are used as a menopausal animal model, as the changes in biochemical and physiological function are comparable to those seen in menopausal women.[20,21,22] The OVX-induced increase in bone loss causes osteoporosis in rats[7] and is similar to that seen in postmenopausal women.[23] Dioscorea has long been used as a Chinese medicine for improving the symptoms seen in the menopause.[5,6] No toxic effect was seen when rats are given dioscorea at a dosage of 500-2000 mg/day for 28 days.[41] Water extracts of dioscorea inhibit the bone resorption induced by parathyroid hormone in a bone culture system.[4] Oral administration of methanol and ethanol extracts of dioscorea have antiosteoporotic activity in OVX rats, enhancing osteoblast differentiation and matrix mineralization,[28] as well as decreasing parathyroid hormone-induced bone resorption.[42] Furthermore, a histomorphometric study using X-ray analysis of the femur in OVX Sprague Dawley rats showed that 33 days treatment with a sustained-released capsule of diosgenin, the major steroidal saponin in dioscorea, reverses the changes in the endosteal perimeter and cortical

area to control levels.[7] The present study showed that oral administration of dioscorea also had antiosteoporotic-like activity, promoting the morphometric and mechanical properties of femora in OVX rats. These results demonstrate that dioscorea and / or its ingredients have beneficial effects on the bone in OVX rats.

Although the stiffness, ultimate force, and toughness of the femora were not affected by ovariectomy, the increase in porosity and decrease in the mineral fraction, indicating a decrease in the quality and quantity of bone,[7,23,43,44] may increase the risk of fracture. The minerals in the bone resist the force acting on the bone and contribute to the mechanical characteristics,[45] which represent the ability of the bone to bear external loads. Chronic dioscorea treatment significantly increased the ultimate force of the femora in OVX rats, suggesting that the mechanical strength was increased. Our previous studies on mechanical tests on the bovine femur demonstrated relationships between the mechanical properties, porosity, and mineral fraction and that the contribution of porosity to the ultimate force was significantly higher than that of the mineral fraction.[18,35] This may explain the observation in the current study that the increase in the ultimate force of the femur in dioscorea-treated OVX rats was accompanied by a decrease in porosity, but no change in the mineral fraction. The effect of dioscorea on bone remodeling during the postmenopause therefore deserves further study.

The sex hormone system may be involved in these effects of dioscorea. Decreased blood levels of sex hormone are thought to be involved in the disorders seen in the postmenopause,[46] as postmenopausal syndrome is significantly improved by hormone replacement therapy.[3] Diosgenin, the main steroidal saponin in dioscorea,[8,9] is used to manufacture steroidal hormones, such as progesterone, estrogen, testosterone, and cortisone,[10,11] by in vitro chemical modification.[12] There are no reports on the exact mechanisms by which diosgenin is converted to other hormones in vivo, but a previous study

showed that hypertrophy of the adrenal cortex in OVX animals was reversed towards control values after continuous supplementation with diosgenin.[47] Furthermore, the consumption of wild Mexican yam products containing diosgenin increases progesterone activity in the saliva,[48] suggesting that the steroidal hormone system is affected. However, it should be noted that a recent study on menopausal animals indicated that sex hormone levels might not be affected by diosgenin treatment.[47] Moreover, dietary supplementation with dioscorea does not affect dehydroepiandrosterone levels in the blood.[49] Thus, dioscorea, and / or diosgenin may not serve as a precursor of sex hormones *in vivo*, but affect menopausal symptoms by another mechanism, for example, an anti-inflammatory action, as dioscorea modulates the production of cytokines *in vivo*[25,50] and *in vitro*.[51] Furthermore, the effects of dioscorea observed in this work may be mediated through its antioxidative activity or by modulating lipid metabolism,[49] as the antioxidative capacity of foods has positive effects on bone.[29] In addition, dioscorea treatment improves nutritional status and the synthesis of proteins and related hormones,[41] in which ingredients of dioscorea (diosgenin and related steroidal saponins) are reported to be involved.[52]

The conditions used in testing, e.g., the dryness, humidity, and age of the bone, the manner in which the force is applied, and whether the bone is kept whole, affect the mechanical characteristics of bones. Because the main goal of this work was not to focus on the characteristics of compact or cancellous bone, but to study the effects of bending force on the whole bone, the whole femur was used for mechanical testing to mimic the situation in which the bone is hit laterally or in a fall. The changes in porosity and bone mineral fraction and their relationship to the mechanical properties seen in this study should not be compared to those for compact or cancellous bones, as the performance of anisotropic bones under axial compression and axial tension differs from that of a long bone under a bending force.

CONCLUSION

In summary, the present data show that ovariectomy causes an increase in porosity and a decrease in bone mineral fraction in the femora of rats. Subsequent chronic administration of dioscorea reverses the effect on porosity and increases the ultimate force. These data provide an insight into the effect of dioscorea on bone remodeling and osteoporosis in the menopause.

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Figure Legends

Fig. 1. Schematic representation of the three-point bending test (A) and the determination of mechanical parameters for the femur using the load-displacement curve (B). F_{ult}, ultimate force; D_{ult}, ultimate displacement.

Fig. 2. Effects of ovariectomy and subsequent dioscorea treatment on changes in body weight. A: Change in body weight in the different groups at 4 weeks after ovariectomy or sham operation expressed as a percentage of the pre-surgery value. The body weight before surgery was 231.7±7.1 g and 272.3±3.5 g in the sham-operated and ovariectomized (OVX) rats, respectively. B: Change in body weight in the different groups over the 4-week period of dioscorea treatment expressed as a percentage of the weight at the start of dioscorea treatment of the sham-operated or OVX rats, as appropriate. The data are shown as the mean ± SEM.

Table

Table 1. Effects of dioscorea on bone properties in sham-operated and OVX rats.

	Sham			OVX			
	0 mg/kg (n=10)	250 mg/kg (n=9)	750 mg/kg (n=11)	0 mg/kg (n=12)	250 mg/kg (n=17)	750 mg/kg (n=16)	1500 mg/kg (n=15)
Porosity (%)	12.7 ± 0.4	12.9 ± 0.7	13.8 ± 0.5	14.0 ± 0.3#	13.3 ± 0.4	12.7 ± 0.3*	12.3 ± 0.3**
Bone mineral fraction (%)	71.3 ± 1.2	71.0 ± 1.2	68.3 ± 0.5	67.8 ± 0.7#	69.4 ± 1.1	70.6 ± 1.1	71.6 ± 1.8
Stiffness (N/mm)	283.9 ± 8.3	283.5 ± 13.2	277.4 ± 9.4	299.0 ± 10.5	316.4 ± 8.8	314.0 ± 6.7	307.4 ± 6.8
Ultimate force (N)	117.4 ± 4.2	113.4 ± 5.3	113.0 ± 4.2	122.9 ± 4.0	135.0 ± 4.2*	139.5 ± 3.2**	136.3 ± 3.2*
Toughness (N·mm)	38.4 ± 2.1	36.7 ± 2.4	37.8 ± 2.2	43.5 ± 2.0	46.5 ± 1.8	48.4 ± 2.0	50.1 ± 2.4

# $P < 0.05$, compared to the sham-operated group receiving 0 mg/kg of dioscorea. * $P < 0.05$, ** $P < 0.01$, compared to the OVX rats receiving 0 mg/kg of dioscorea. The data are the mean ± SEM for the number of rats indicated in parentheses.

Fig. 1.

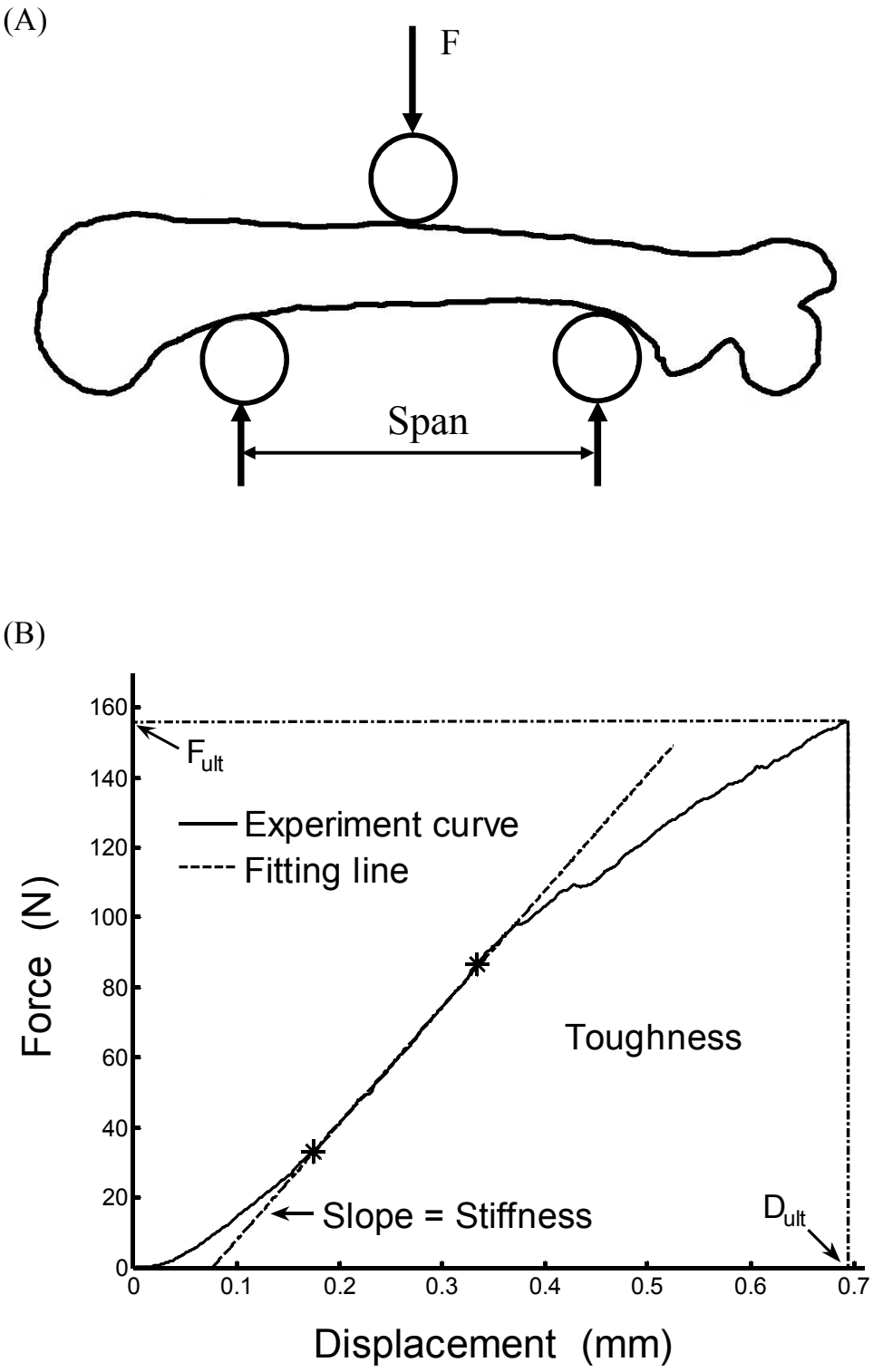
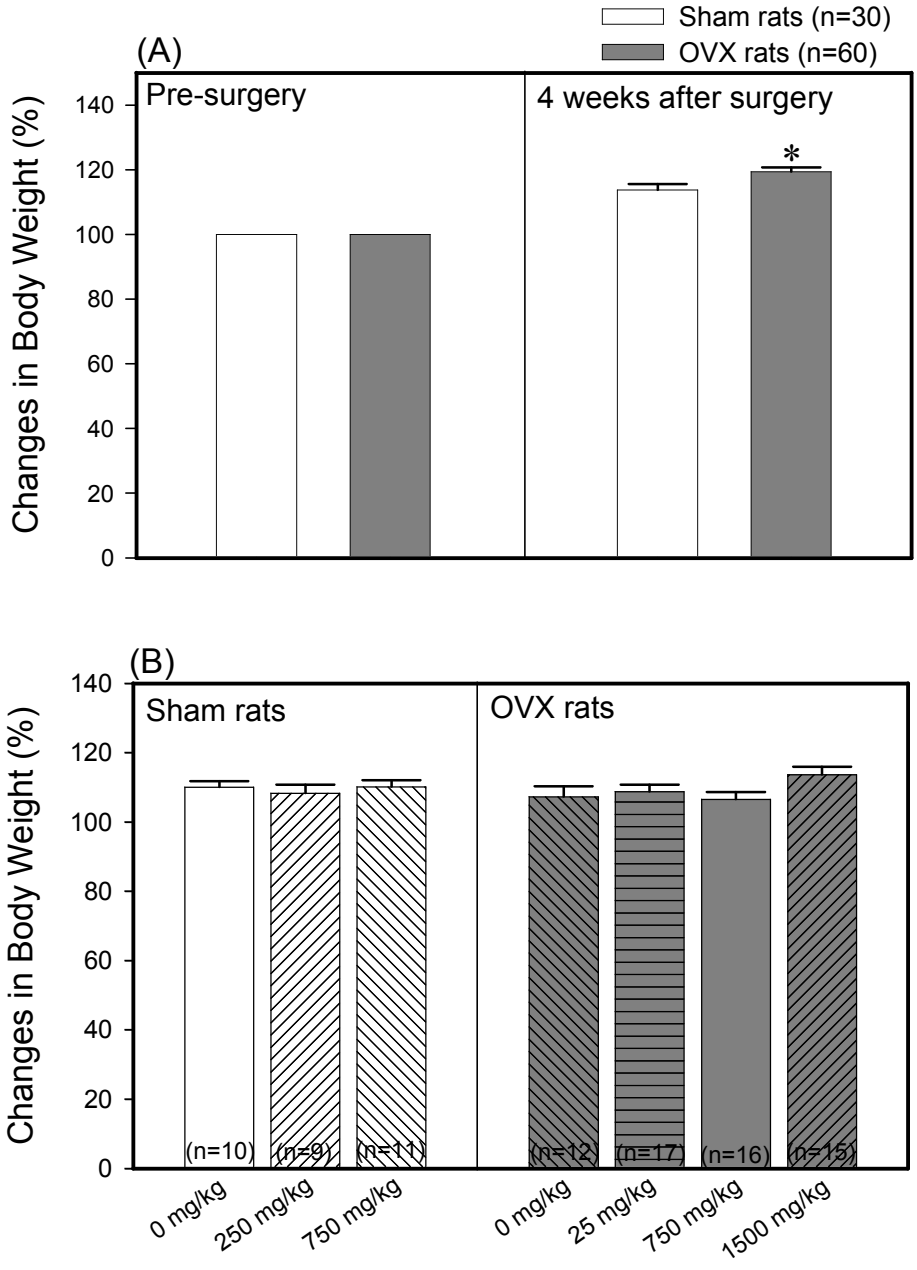

(A)
F
Span
(B)
160
140
120
100
80
60
40
20
0
F_{ult}
Experiment curve
Fitting line
Force (N)
Toughness
Slope = Stiffness
D_{ult}
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Displacement (mm)

Figure-2

Fig. 2.



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