

**TNF- α MEDIATES OSTEOPENIA CAUSED BY DEPLETION OF
ANTIOXIDANTS**

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ABSTRACT

We recently found that estrogen deficiency leads to a lowering of thiol antioxidant defenses in rodent bone. Moreover, administration of agents that increase the concentration in bone of glutathione, the main intracellular antioxidant, prevented estrogen-deficiency bone loss, while depletion of glutathione by buthionine sulfoximine (BSO) administration provoked substantial bone loss. It has been shown that the estrogen-deficiency bone loss is dependent upon TNF- α signaling. Therefore, a model in which estrogen-deficiency causes bone loss by lowering antioxidant defenses predicts that the osteopenia caused by lowering antioxidant defenses should similarly depend on TNF- α signaling. We found that the loss of bone caused by either BSO administration or ovariectomy was inhibited by administration of soluble TNF- α receptors, and abrogated in mice deleted for TNF- α gene expression. In both circumstances, lack of TNF- α signaling prevented the increase in bone resorption and the deficit in bone formation that otherwise occurred. Thus, depletion of thiol antioxidants by BSO, like ovariectomy, causes bone loss through TNF- α signaling. Furthermore, in ovariectomized mice treated with soluble TNF- α receptors, thiol antioxidant defenses in bone remained low, despite inhibition of bone loss. This suggests that the low levels of antioxidants in bone seen after ovariectomy are the cause, rather than the effect, of the increased resorption. These experiments are consistent with a model for estrogen-deficiency bone loss in which estrogen deficiency lowers thiol antioxidant defenses in bone cells, thereby increasing ROS levels, which in turn induce expression of TNF- α , which causes loss of bone.

INTRODUCTION.

Postmenopausal osteoporosis is a disease of high prevalence and a cause of substantial morbidity and mortality. It is caused by estrogen deficiency, which leads to bone loss through increased osteoclastic function, combined with a relative deficiency of osteoblastic function, whereby osteoblasts fail to compensate for the increase in bone resorption (1). The mechanisms through which estrogen deficiency stimulates bone resorption and impairs bone formation remain controversial. Several mechanisms have been suggested to account for the increase in resorption, including direct effects of estrogen on osteoclasts (2-4), upregulation of the osteoclast-inductive cytokine RANK ligand (RANKL) (5), and downregulation of its decoy receptor osteoprotegerin in osteoblasts (6). In addition, several inflammatory cytokines have been implicated in the bone loss: estrogen-deficiency has been reported to cause bone loss through increased expression of TNF- α , IL-1 and IL-6 in osteoclast-supportive bone marrow stromal cells, monocytes, and lymphocytes (see 1, 7, 8 for reviews).

We recently found that signaling through reactive oxygen species (ROS) might be the mechanism through which deficiency of estrogen causes bone loss: ovariectomy in rodents lowered antioxidant defenses in bone; and antioxidant defenses were rapidly restored by estrogen replacement. Moreover, administration of antioxidants that increase the intracellular concentration of glutathione, the major intracellular antioxidant, prevented ovariectomy-induced osteopenia, while administration of buthionine sulfoximine (BSO), which depletes intracellular glutathione (9), caused bone loss.

The mechanism through which ROS cause loss of bone is uncertain. Amongst ROS, hydrogen peroxide is the most likely species to act as an intra- or intercellular signal, since it has a relatively long half-life and is membrane permeant. It has moreover been shown to directly stimulate osteoclast formation and function (10, 11). In addition, hydrogen peroxide stimulates expression of TNF- α , a cytokine implicated in the bone loss caused by estrogen deficiency, in many cells; and large amounts of hydrogen peroxide are generated by resorbing osteoclasts (12)

If estrogen-deficiency does cause bone loss by lowering antioxidant defenses, we would predict that the loss of bone induced by lowering of antioxidant defenses is, like that of estrogen-deficiency, dependent upon TNF- α signaling. It is known that TNF- α expression can be induced by ROS. If ROS cause bone loss in ovariectomised mice through induction of TNF- α expression, the bone loss observed in BSO-treated mice should be similarly dependent upon TNF- α : BSO is a specific inhibitor of glutathione synthesis, and administration of BSO leads to increased intracellular ROS and augmented TNF- α expression (9, 13-15). We therefore tested the ability of BSO administration to induce bone loss in mice treated with soluble TNF- α receptors, and in mice deleted for TNF- α expression. We found that BSO was unable to induce bone loss under these circumstances. These results are consistent with the notion that estrogen-deficiency lowers antioxidant levels in bone, and that this causes a ROS-mediated increase in TNF- α , which causes the bone loss.

MATERIALS AND METHODS

Media and Reagents

Soluble TNF-receptor I (TNFSR) was donated by Amgen (Thousand Oaks, Ca). All other reagents were obtained from Sigma unless otherwise stated.

Animals

B6;129S6 mice deleted for TNF- α expression (16) and B6;129SF2 wild-type mice were obtained from The Jackson Laboratories, Bar Harbor, Maine. These mice (backcross generation N1F3) were 8 wk old at the time of the experiments. Female 6-8-wk-old MF1 mice were obtained from Harlan Olac (Oxon, UK). All experiments were conducted with the approval of the Home Office.

Assessment of the effect of TNF- α expression on bone loss caused by BSO-treatment or ovariectomy.

To assess the effect of blockade of TNF- α signaling using soluble receptors to TNF- α , groups of 6 female MF1 mice were administered BSO (2 mmol/kg *ip*) twice per day (7.00am and 5.00pm) for 2 wks. For these groups, BSO was also included in the drinking water (20 mM). Further groups were subjected to ovariectomy or sham-ovariectomy. Mice were also administered TNFSR (2.5 mg/kg, *ip*) or vehicle daily. Mice were weighed before and after the experiments. All animals were pair-fed. Calcein (30 mg/kg) was injected *ip* 1 and 3 days before killing. Animals were killed after 2 wks. Success of ovariectomy was confirmed by absence of ovaries and atrophy of uteri. Femora were

removed and cleaned of soft tissue. One femur from each animal was fixed in 70% alcohol for 48h, dehydrated through graded alcohols and embedded in London Resin (London Resin Co Ltd, Basingstoke, UK). Fluorochrome labeling was assessed in unstained sections. For static histomorphometric analysis, the second femur was fixed for 24 hrs in 10% phosphate buffered formalin, demineralized in 10% buffered EDTA for 7 days, dehydrated through graded alcohols and embedded in paraffin wax. Sections were prepared and submitted to histomorphometric analysis as described (17).

Bones from ovariectomised mice treated with soluble receptors or vehicle, and sham-operated control mice were also used to measure glutathione content. For this, femora were rapidly cleaned and bone marrow harvested into ice-cold heparinized water and homogenized by repeatedly passing through a 23g needle. The homogenates were divided into two equal parts. To one, 0.1 vol of 1% Triton-X100 was added; to the other an equal volume of 10% sulfosalicylic acid. The Triton extract was centrifuged at 10,000 g for 10 min at 4°C and the supernatant was used for enzyme assays. Protein concentration was determined using Coomassie blue (Pierce, Tattenhall, Cheshire, UK) with bovine serum albumin as a standard. Glutathione was measured in the samples after deproteinization with sulfosalicylic acid. Total glutathione (GSH + GSSG) was measured using the GSH reductase-DTNB recycling procedure according to Tietze (18). GSSG was assayed as above after derivatization of GSH in the sample with 2-vinylpyridine (18).

To further assess the role of TNF- α in BSO-mediated and estrogen-deficiency bone loss, groups of 6 TNF-deleted or wild-type mice were treated with BSO, or subjected to

ovariectomy or a sham operation, as described above. All animals were killed 2 wks later, and bones were prepared and processed for histomorphometric analysis as described above.

Statistical analysis was by ANOVA (Fisher's PLSD test) for multiple comparisons, and Student's t-test for paired comparisons.

RESULTS

As previously noted, BSO caused, like ovariectomy, a significant decrease in bone volume (Fig 1 and 2A). Also like ovariectomy, bone loss was associated with an increase in the indices of bone resorption (Fig 2B-D). Ovariectomy caused an increase in the static and dynamic indices of bone formation (Fig 2E-I). This is a well-recognized phenomenon, and is due to the coupling of bone resorption and formation, whereby an increase in bone resorption leads to a coupled increase in bone formation. Net loss of bone occurs after ovariectomy because bone formation, although increased, does not match the increase in bone resorption. In mice treated with BSO, bone loss was accompanied by a reduction in the extent of bone surface covered by osteoblasts (Fig 2E), and the number of osteoblasts (Fig 2F), and in the histodynamic indices of bone formation (Fig 2G-I). This suggests that in both BSO-treated and estrogen deficient mice, bone formation does not fully compensate for the increase in resorption. However, compensation is lower in BSO-treated mice than in those with estrogen-deficiency.

TNFSR did not alter any of the indices of bone resorption or bone formation in wild-type mice (Fig 2). This is consistent with the observation that mice deficient for TNF- α signaling have normal bones (19). However, the soluble receptors completely prevented the bone loss caused by BSO. Similarly, while significant bone loss was observed in ovariectomized mice treated with soluble receptors, bone volume was significantly greater in these mice than in untreated ovariectomized mice (Fig 2), suggesting a partial inhibition of ovariectomy-induced bone loss by the soluble receptors. Also consistent

with modulation of ovariectomy-induced bone loss by the soluble receptors, the increases in osteoclast and osteoblast indices caused by ovariectomy were all normalized by TNFSR (Fig 2). TNFSR also suppressed the BSO-mediated increase in indices of bone resorption, although indices of bone formation remained significantly inhibited. This suggests that a component of the osteoblastic defect caused by BSO is not mediated by TNF- α .

We noted that BSO caused significant weight loss in the mice (Table). The mice appeared healthy. BSO causes depletion of cellular glutathione, which causes oxidative stress. Thus, its effects can be neutralized by antioxidants (9, 20). Glutathione depletion suppresses differentiation (21), so that the weight loss might reflect a more generalized suppression of differentiated cell function, not limited to osteoblasts, by BSO-induced glutathione depletion. Because BSO affects all cells, it is to be expected that its effect will be more widespread than that caused by estrogen-deficiency. Whatever the explanation for the weight loss, our results show that TNF signaling is required for the enhanced osteoclastic activity and loss of bone caused by BSO.

We assessed further whether BSO-induced bone loss is mediated by TNF- α using mice in which the gene for TNF- α is deleted (16). The bones of these mice showed a very similar response to BSO and ovariectomy to that observed in mice treated with TNFSR (Fig 3): while wild-type mice lost bone with BSO or after ovariectomy, mice deleted for TNF- α expression showed no significant bone loss (Fig 3A). After BSO administration or ovariectomy, wild-type mice showed the expected increases in the indices of bone

resorption (Fig 3B-D). In contrast, resorption parameters were unaffected by either BSO or ovariectomy in TNF- α deficient mice (Fig 3B-D). Indices of bone formation (Fig 3E-I) were suppressed by ovariectomy in wild-type mice, consistent with the defective ability of osteoblasts to compensate for bone resorption in estrogen-deficient states: this ability is likely to differ between strains, since mice of differing genetic background show intrinsic differences in osteoblastic activity (22,23). BSO similarly suppressed bone formation indices. In contrast, bone formation indices were unaffected by either BSO or ovariectomy in mice deficient for TNF- α (Fig 3E-I).

As previously reported, bone marrow glutathione levels were decreased by ovariectomy (Fig 4). We noted that ovariectomised mice administered with TNFSR also showed a decrease in glutathione. This suggests that the lower glutathione concentration in the bone marrow of ovariectomised mice is the cause rather than the effect of increased bone resorption.

DISCUSSION

We have previously found that estrogen deficiency causes a lowering of antioxidant defenses in bone, and that while antioxidants prevent estrogen-deficient bone loss, lowering of oxidant defenses causes osteopenia. We now report that bone loss due to the lowering of antioxidant defenses causes bone loss, like ovariectomy, through a TNF- α -dependent mechanism. We also found that antioxidant defenses are lowered after ovariectomy even if bone loss is prevented, establishing that lowering of thiol antioxidant defenses is the cause, rather than the effect, of the bone loss.

The induction of expression of TNF- α is a common consequence of the exposure of cells to ROS. This cytokine has also been implicated in the bone loss that follows ovariectomy (24, 25). Thus, if estrogen-deficiency causes bone loss through ROS, we would predict that the bone loss induced by the lowering of oxidant defenses by BSO would, like that of estrogen-deficiency, be dependent on TNF- α . We found clear evidence that BSO-mediated bone loss was indeed dependent upon TNF- α : bone loss was substantially reduced by administration of soluble receptors for TNF- α ; and BSO did not cause bone loss in mice from which the TNF- α gene was deleted.

Although ROS are well known to be capable of inducing TNF- α expression, many of the signaling systems implicated in osteoclastic differentiation, especially AP-1, NF κ B, PI3K and p38 MAP kinase, are also targets for ROS. Therefore, ROS might augment osteoclast formation by direct actions on the intracellular signaling systems that are responsible for

osteoclast formation. However, many of the signals necessary for osteoclast formation, including the activation of NF κ B by BSO in osteoclasts we previously observed (11), are also implicated in the induction of TNF- α expression by ROS. The dependence of ROS-induced bone loss on TNF- α that we now report suggests that ROS do not increase bone resorption in vivo by directly augmenting the intracellular signals that stimulate osteoclastic differentiation or activity, but rather act by augmenting intracellular signals that stimulate TNF- α expression. It may be that while osteoclast formation is dependent upon AP-1 and NF κ B signaling, TNF- α expression is dependent upon the magnitude of these signals.

Recently, it was shown that TNF- α synergises strongly with RANKL for osteoclast formation and activation (26, 27). Therefore, ROS might induce bone hyper-resorption through the direct autocrine-paracrine effects of ROS-induced TNF- α expression in osteoclasts. This does not preclude, however, similar induction of TNF- α expression by ROS in osteoblastic, endothelial, macrophagic, lymphoid and other cells in the bone micro-environment. Nor does it preclude effects of TNF- α on osteoclast-inductive signaling by osteoblastic and other cells. Furthermore, ROS and TNF- α not only stimulate resorption but also suppress osteoblastic differentiation (28-31). Thus, the induction of TNF- α by ROS might contribute to the defect in osteoblastic function that is observed in estrogen-deficient states.

In addition to the similarities between BSO-induced and ovariectomy-induced bone loss, we also noted differences. Although both BSO and ovariectomy caused bone loss through

increased bone resorption that was not compensated for by bone formation, BSO caused a relatively greater deficiency in osteoblast function than was observed in ovariectomised mice. The osteoblastic deficit caused by BSO was completely absent from mice deleted for TNF- α , but was only partly prevented by the soluble receptors. This suggests that the osteoblastic defect occurs largely if not completely through the same mechanism as that which causes the osteoblast defect of ovariectomy, ie through TNF- α expression. We previously noted (10) that estrogen modulates thiol antioxidants in osteoclasts but not osteoblastic cells, while BSO will suppress glutathione levels in all cells. We would therefore anticipate that BSO might cause a relatively greater effect on osteoblast function than would ovariectomy.

The identity of the ROS responsible is uncertain. Amongst ROS, hydrogen peroxide is the most likely species to act as an intra- or intercellular signal, since it is sufficiently long-lived and membrane permeant to transmit ROS signals to nearby cells. It has moreover been shown to directly stimulate osteoclast formation and function (10, 11). In addition, hydrogen peroxide stimulates expression of TNF- α , in many cells; and large amounts of hydrogen peroxide are generated by resorbing osteoclasts (12). Moreover, we have recently found that glutathione peroxidase, the glutathione-dependent enzyme most effective in the degradation of hydrogen peroxide, is also the antioxidant enzyme that is most highly expressed by osteoclasts, and its expression by osteoclasts is stimulated by estrogen (unpublished). We would therefore anticipate that hydrogen peroxide concentrations might increase in the bone micro-environment after ovariectomy.

Whatever the identity of the ROS, and of the cell that generates and responds to ROS in bone, our results show that ROS, like ovariectomy, cause bone loss through TNF- α signaling. This implies a model for bone loss in which estrogen deficiency lowers antioxidant defenses in bone, and thereby increases ROS levels. This induces expression of TNF- α , which causes osteoclastic hyper-resorption and loss of bone.

FIGURE LEGENDS

Figure 1. Soluble TNF- α receptors inhibit the bone loss induced by BSO and ovariectomy. Representative sections of femora from mice ovariectomized or treated with BSO (2mmol/kg ip) twice per day for 2 weeks. BSO was also included in the drinking water (20 mM). Mice were also administered soluble receptors for TNF- α (TNFSR) (2.5 mg/kg) or vehicle daily.

Figure 2. Soluble TNF- α receptors inhibit the bone loss induced by BSO and ovariectomy. Mice were ovariectomized or injected with BSO (2 mmol/kg ip) twice per day for 2 weeks. BSO was also included in the drinking water (20 mM). Mice were also administered soluble receptors for TNF- α (TNFSR) (2.5 mg/kg) or vehicle daily, ip. A: the quantity of trabecular bone was measured as a percentage of the area occupied by trabecular bone, as a percentage of the total area. B-D (see ref 17): indices of osteoclastic function. B: ES/BS: percentage of bone surface showing an eroded appearance; C: oc surface: the percentage of bone surfaces covered by osteoclasts; D: oc no: the number of osteoclasts on bone surfaces. E-F: static measurements of osteoblastic function, derived from measurements of: E: ob surface: the percentage of bone surfaces covered by osteoblasts; F: ob no: the number of osteoblasts on bone surfaces. G-I: dynamic measures of osteoblastic function, derived from analysis of fluorochrome-labeled resin sections of bones. G: dLS/BS: the proportion of bone surface that shows

double fluorochrome labeling; H: MAR: mineral apposition rate, the distance between two fluorochrome labels, per unit time; I: BFR: bone formation rate, expressed as the volume of bone formed per unit of bone surface, derived from dLS and MAR. Symbols below the bar diagrams refer to plus/minus TNFSR.

* $p < 0.05$ vs sham; ^a $p < 0.05$ vs ovx.

Figure 3. BSO and ovariectomy do not cause bone loss in mice deleted for TNF- α expression. Mice expressing (+) or deleted for (-) TNF- α were ovariectomised or injected with BSO (2 mmol/kg ip) twice per day for 2 weeks. BSO was also included in the drinking water (20 mM). A: the quantity of trabecular bone was measured as the area occupied by trabecular bone, as a percentage of the total area. B-D: indices of osteoclastic function. B: ES/BS: percentage of bone surface showing an eroded appearance; C: oc surface: the percentage of bone surfaces covered by osteoclasts; D: oc no: the number of osteoclasts on bone surfaces. E-F: static measurements of osteoblastic function, derived from measurements of: E: ob surface: the percentage of bone surfaces covered by osteoblasts; F: ob no: the number of osteoblasts on bone surfaces. G-I: dynamic measures of osteoblastic function, derived from analysis of fluorochrome-labeled resin sections of bones. G: dLS/BS: the proportion of bone surface that shows double fluorochrome labeling; H: MAR: mineral apposition rate, the distance between two fluorochrome labels, per unit time; I: BFR: bone formation

rate, expressed as the volume of bone formed per unit of bone surface, derived from dLS and MAR. Symbols below the bar diagrams refer to wild-type mice (+) and mice deleted for TNF- α -expression (-).

*p<0.05 vs sham.

Figure 4. Assessment of the effect of soluble receptors for TNF- α on bone marrow glutathione content. Mice were ovariectomized or sham-ovariectomised, and injected daily with soluble receptors for TNF- α . The concentration of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) in the bone marrow was then measured.

*p<0.05 vs sham-ovariectomised group.

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Experiment to test effect of TNFSR			Experiment to test effect of TNF- α ko		
<i>body weight (g)</i>	<i>pre</i>	<i>post</i>		<i>pre</i>	<i>post</i>
sham ovx	29.5 \pm 0.9	29.9 \pm 0.7	sham wt	19.2 \pm 0.5	20.3 \pm 0.3
BSO	29.0 \pm 0.9	*26.4 \pm 0.6 ^a	BSO wt	19.3 \pm 0.8	*18.0 \pm 0.6
ovx	28.5 \pm 3.7	30.5 \pm 0.9	ovx wt	18.3 \pm 0.6	20.7 \pm 0.9
TNFSR	28.9 \pm 0.9	29.7 \pm 0.7	sham ko	18.8 \pm 0.5	20.6 \pm 0.5
BSO/TNFSR	28.0 \pm 0.7	*25.4 \pm 0.5 ^a	BSO ko	18.4 \pm 0.4	*18.1 \pm 0.8
ovx/TNFSR	28.0 \pm 0.7	28.4 \pm 0.4	ovx ko	16.1 \pm 0.6	20.9 \pm 0.9

Table. Body weight of mice before and after experiments to test the effect of soluble TNF receptors (TNFSR) and TNF- α gene deletion (TNF- α -ko) on bone loss caused by BSO treatment and ovariectomy. * p <0.05 vs appropriate sham-ovariectomized group. ^a: p <0.05 vs body weight prior to experiment. wt: wild-type.

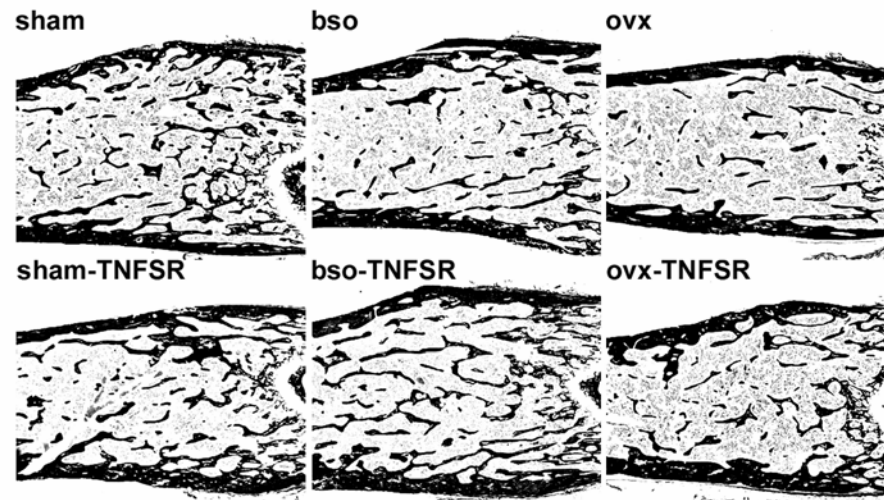


Figure 1
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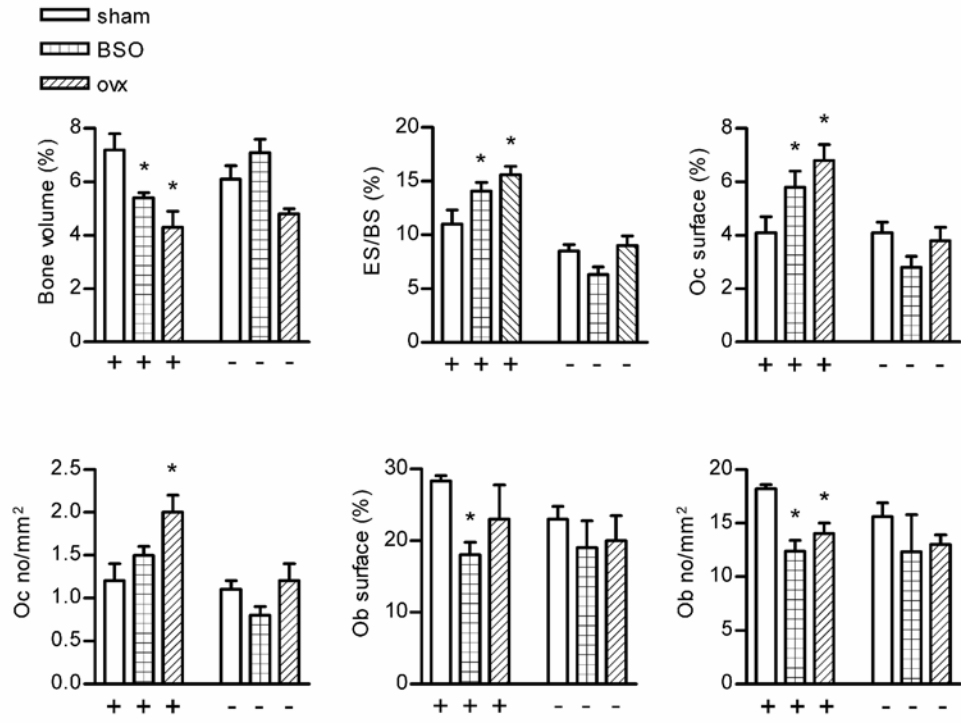


Figure 2
 Jagger et al

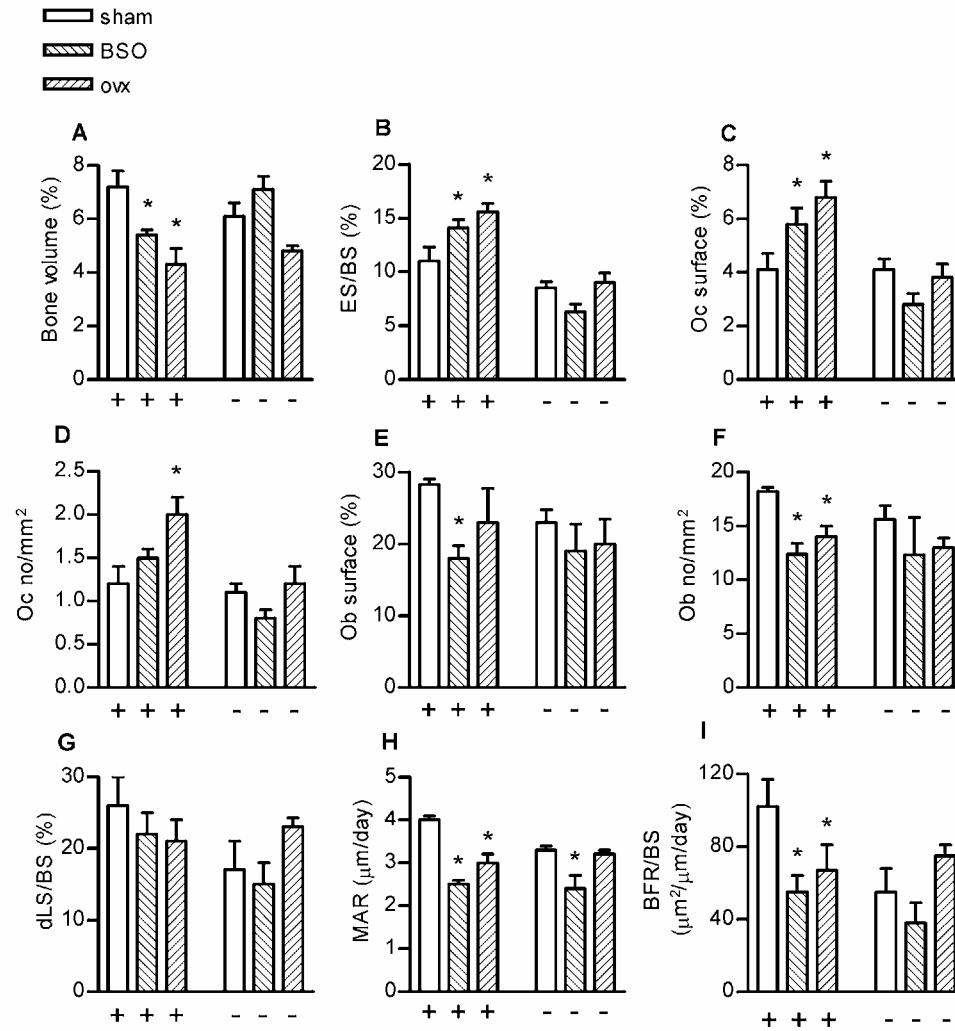


Figure 3
 Jagger et al

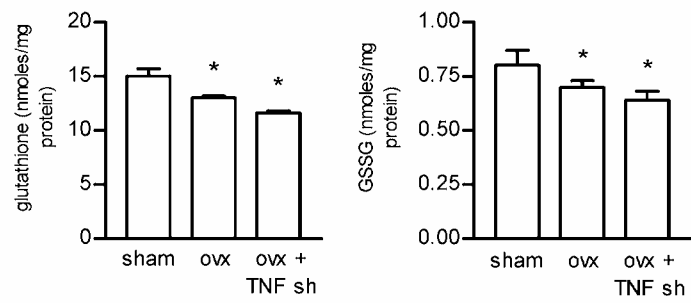


Figure 4
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