INTRODUCTION

Osteoporosis is accepted as a major public health problem characterized by compromised bone strength, micro-architectural deterioration of bone tissue and increased fracture risk with an important socio-economic burden. It is a multifactorial disease, but the most common form is involutional osteoporosis, which is associated with advancing age and menopause. Along with efforts to develop improved antiresorptive agents, there has been a long-standing goal to develop therapeutics that can stimulate bone formation, to increase bone mass, and strength for low bone mass conditions (e.g. osteoporosis).

Wnt proteins are signaling molecules that regulate osteoblasts differentiation into mature osteoblasts. The binding of Wnt proteins to their specific receptors leads to stabilization of β-catenin, which translocates to the nucleus and regulates gene expression. Sclerostin is a natural Wnt antagonist secreted by osteocytes. Sclerostin antibodies (Scl-Abs) have been shown to neutralize the inhibitory effects of sclerostin on Wnt/b-catenin signaling and might be an attractive approach for the development of a novel bone anabolic agent.

Estrogen (E2) is involved in the regulation of a number of molecules that have an effect on osteoclasts. E2 limits the size of preosteoclast population by apoptosis and stimulates osteoblastic stromal cells to synthesize more osteoprotegerine (OPG) that inhibits osteoclast differentiation and bone resorption. E2 also stimulates osteoblastic cells to make Transforming Growth Factor-beta (TGF β) which restrains the expression of cathepsin K, which is the molecular shovel that osteoclasts used to dig holes in bone.

Ovariectomized (OVX) rat models, mimic changes in bone metabolism observed in postmenopausal osteoporosis and have been most commonly used for the efficacious studies of potential therapeutic agents for the treatment of osteoporosis.

The aim of this study was to determine the systemic effect of sclerostin antibody administration on markers of bone formation in rats made estrogen deficient (OVX) and to compare this effect with that of raloxifene and a combination of sclerostin antibody and raloxifene.
METHODOLOGY

Forty-five female Wistar rats were housed at room temperature (25±°C) and were allowed water ad libitum. Their initial weight varied from 225 to 250 grams and age from 19 to 20 weeks. The experiments were conducted in accordance with Institutional Review Board (IRB) at King Khaled University Hospital, Riyadh. The rats were divided randomly into five groups equally. Group I included sham operated (intact) control rats. Group II, III, IV and V were OVX rats received saline, sclerostin monoclonal antibody III (Scl-AbIII), raloxifene or a combination therapy of both Scl-AbIII and raloxifene, respectively.

Rats were anesthetized using ketamine hydrochloride (120 mg/kg) and xylazine hydrochloride (24 mg/kg) intramuscularly.9 The experiment for bilateral ovariectomy was done through abdominal incision to expose the uterus, oviducts and ovaries. The oviduct and blood vessels supplying the ovaries were tied up, and the ovaries were removed through an incision. The uterus and oviducts were put back with the fat tissues around them, and the incision was closed with suture. In sham operated rats, an abdominal incision was performed affecting skin and muscle and peritoneum. No organ was extripated or handled. The wound was subsequently sealed. The animals were randomized into groups according to the above protocol.

Treatments of OVX rats were started one month post-surgery. Twenty-five mg/kg of Scl-AbIII was injected subcutaneous (S.C.) 2 times per week for 4 weeks.8 Scl-AbIII stock solution was stored at -80°C. The dosing solutions were prepared weekly by completely thawing in a 4°C refrigerator overnight then diluting with saline to a concentration of 5 mg/ml. Raloxifene was purchased from Sigma Aldrich Co. and administered orally at a daily dose of 5mg/kg, for 4 weeks.9 Body weight was measured at the start and at the end of the experiment. Body weight change was calculated for each rat.

At the end of the treatment, blood samples (by cardiac puncture) were collected at 7:00 and 9:00 A.M., centrifuged and frozen within one hour, and stored under identical conditions. The serum samples were assayed for the levels of estradiol by competitive enzyme immunoassay.10 Bone specific alkaline phosphatase (BSAP) using immunoradiometric assay,11 alkaline phosphates by colorimetric method,12 osteocalcin by immunoassay,11 insulin like growth factor-1 (IGF-1) by enzyme linked immunosorbent assay (EASIA kit),13 parathyroid hormone (PTH) by enzyme amplifier sensitivity immunoassay (EASIA),14 and calcium and phosphorous by colorimetric method.15 Uteri were excised from sacrificed animals and weighed after trimming associated fat and expressing any luminal fluid.

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) program for windows version 21. Data were expressed as mean ±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with 95% confidence intervals followed by Tukey's multiple comparison tests. Differences were considered to be statistically significant at p < 0.05.

RESULTS

OVX-control rats had statistically significant decrease of estradiol level in comparison with the sham-operated controls. Neither Scl-AbIII nor raloxifene administration affected the decrease in estradiol levels (Table I). Both serum BSAP and alkaline phosphatase levels were significantly decreased in control OVX rats as compared to sham-operated controls and OVX rats treated with Scl-AbIII, raloxifene or Scl-AbIII+Raloxifene (p < 0.001). Scl-AbIII administration for four weeks increased bone formation markers, as demonstrated by a 19.8% increase in serum BSAP and a 24.8% increase in serum alkaline phosphatase as compared to control OVX rats (p < 0.001). Similar results were found after 4 weeks of raloxifene treatment. Combined Scl-AbIII+raloxifene treatment resulted in greater increase in serum BSAP and alkaline phosphatase (42.2%, 44.5% respectively

Table I: Mean Values ± SD of the measured parameters in controls (group I), ovariectomized rats (OVX) rats (group II), sclerostin antibody III (Scl-AbIII)-treated OVX rats (group III), Raloxifene-treated OVX rats (group IV), and OVX rats treated with both Scl-AbIII and raloxifene (group V).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum estradiol (pmol/l)</td>
<td>20.70 ±1.76</td>
<td>11.17 ±0.98*</td>
<td>11.06 ±0.94*</td>
<td>10.94 ±0.59*</td>
<td>10.91 ±0.71*</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum alkaline phosphates (U/l)</td>
<td>351.18 ±43.48</td>
<td>254.18 ±26.77†</td>
<td>317.09 ±28.82</td>
<td>318.67 ±26.81</td>
<td>367.37 ±22.27‡‡</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum osteocalcin (ng/ml)</td>
<td>3.86 ±0.30</td>
<td>2.61 ±0.27††</td>
<td>3.67 ±0.47</td>
<td>3.86 ±0.27</td>
<td>4.38 ±0.44‡</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum insulin like growth factor-1 (ng/ml)</td>
<td>268.02 ±8.40</td>
<td>236.33 ±10.76†</td>
<td>263.55 ±2.86</td>
<td>266.62 ±9.68</td>
<td>280.16 ±7.06§‡‡</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum parathyroid hormone (pg/ml)</td>
<td>34.62 ±2.17</td>
<td>28.99 ±3.19†</td>
<td>34.10 ±2.33</td>
<td>35.73 ±3.20</td>
<td>36.36 ±3.30</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum calcium (mmol/l)</td>
<td>3.53 ±0.51</td>
<td>2.96 ±0.28††</td>
<td>3.57 ±0.37</td>
<td>3.60 ±0.41</td>
<td>3.59 ±0.37</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum phosphorus (mmol/l)</td>
<td>3.65 ±0.28</td>
<td>4.18 ±0.58‡</td>
<td>3.49 ±0.29</td>
<td>3.81 ±0.31</td>
<td>3.69 ±0.33</td>
<td>0.003</td>
</tr>
<tr>
<td>Body weight changes (g)</td>
<td>76.07 ±2.52</td>
<td>99.61 ±2.50‡</td>
<td>98.81 ±2.15**</td>
<td>74.58 ±3.45</td>
<td>72.73 ±3.36</td>
<td>0.000</td>
</tr>
<tr>
<td>Uterus wet weight (g)</td>
<td>536.44 ±15.31</td>
<td>128.33 ±4.21</td>
<td>129.44 ±4.25</td>
<td>198.33 ±4.36§§</td>
<td>199.11 ±4.96‡‡</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Significance was considered at p<0.05, 95% confidence interval.

(†) p<0.001 versus group II; (‡) p<0.001 versus groups II, III, IV and V; (♀) p<0.01 versus group I; (§) p<0.05 versus group III; p<0.05 versus group IV; (§§) p<0.05 versus groups I (p<0.05), III (p<0.05), IV (p<0.05); (††) p<0.05 versus groups I, III, IV and V; (**) p<0.001 versus groups I, IV and V; (***) p<0.001 versus group I.
Effect of sclerostin antibody III (Scl-AbIII) and raloxifene on serum bone specific alkaline phosphatase in controls (group I), ovariectomized rats (OVX) rats (group II), sclerostin antibody III (Scl-AbIII)-treated OVX rats (group III), Raloxifene-treated OVX rats (group IV) and OVX rats treated with both Scl-AbII and raloxifene (group V). All values are represented as mean±S.D. * = p<0.001 versus all other groups, £ = p<0.001 versus groups III and IV.

Control OVX rats had significantly lower serum osteocalcin levels as compared to sham-operated controls. Combined Scl-AbIII+raloxifene-treated rats had higher BSAP and alkaline phosphatase levels as compared to rats treated by either Scl-AbIII or raloxifene alone (Table I, Figure 1).

The decrease in serum level of IGF-1 was evident in control OVX rats as compared to sham-operated controls and OVX rats treated by either Scl-AbIII or raloxifene alone or combined. Combined treatment of OVX rats with Scl-AbIII and raloxifene significantly increased IGF-1 level as compared to sham-operated controls (p < 0.01) or raloxifene (p < 0.05) each separately. The increases in osteocalcin levels were 67.8%, 47.9%, 40.6% higher in Scl-AbIII+raloxifene (gp V), raloxifene (gp IV) and Scl-AbII-treated rats (gp III) respectively as compared to control OVX rats (p < 0.001). No significant change could be detected between Scl-AbII-treated rats and raloxifene-treated rats (Table I).

Control OVX rats showed significant decrease of serum PTH and Ca²⁺ levels and significant increase of serum phosphorous levels as compared to sham-operated controls and OVX rats treated with either Scl-AbIII, raloxifene or Scl-AbIII+raloxifene. PTH levels increased significantly with Scl-AbIII (26.34%), raloxifene (32.38%) or Scl-AbIII+raloxifene (34.72%) as compared to control OVX rats (p < 0.001). Combined treatment of OVX rats with Scl-AbIII+raloxifene did not affect the increase of Ca²⁺ or the decrease of phosphorous levels achieved by treatment of either of Scl-AbIII or raloxifene alone (Table I).

After 4 weeks of observation, body weight increased in all groups. However, when comparing the different study groups, the weight gain of control OVX rats and those rats treated with Scl-AbIII rats at the end of the study period was more pronounced than that of sham-operated controls (99.61 ±2.50, 98.81 ±2.15 vs. 76.07 ±2.52 g, respectively). Raloxifene therapy, either alone or combined with Scl-AbIII provided to OVX rats, kept weight changes comparable with those seen in sham-operated controls (74.58 ±3.45, 72.73 ±3.36 vs. 76.07 ±2.52 g, respectively, Table I).

Bilateral ovariectomy induced a substantial decrease of uterus wet weight in relation to the sham-operated controls, expected results of a lowered E2 level, and was 23.92% of the uterine weight of the intact control. Although administration of Scl-AbIII to OVX rats did not affect uterine wet weight; administration of raloxifene, either alone or combined with Scl-AbIII, attenuated the decrease of uterus mass in the OVX rats (36.97%, 37.12% respectively of the uterine weight of the sham-operated controls (Table I).

DISCUSSION

The present study showed an increase in serum BSAP and alkaline phosphatase after 4 weeks’ administration of either Scl-AbIII or raloxifene. While, combined treatment with Scl-AbIII and raloxifene resulted in more increase in BSAP and serum alkaline phosphatase as compared to non-treated OVX rats (p < 0.001).

The marked improvements in bone formation markers in Scl-AbIII-treated OVX rats provide the evidence that inhibition of sclerostin can enhance bone formation, which may be directly related to the effects of reversing sclerostin’s inhibitory effect on osteoblast activity. The mechanism by which sclerostin negatively regulates bone formation is an area of continuing investigation. One body of research supports the hypothesis that sclerostin inhibits Wnt-β-catenin signaling by interacting with Wnt coreceptors and thus impairing osteoblast differentiation and function. Consistent with the present findings is the human clinical study of Padhi et al. who found that a single dose of a humanized sclerostin monoclonal antibody in healthy men and postmenopausal women resulted in a dose-dependent increase in the concentrations of the bone-formation markers (BSAP and osteocalcin) and decreased the bone-resorption marker (serum C-telopeptide). In addition, Tasci et al. found high serum alkaline phosphatase in raloxifene treated osteoporotic rats.

Serum levels of osteocalcin are regarded as sensitive and specific marker of osteoblastic activity and the rate of bone formation. Hence, the finding of the present
work of reduced serum osteocalcin in OVX rats suggests that reduced osteoblast activity may be responsible for the osteoporosis. Evidence of a stimulatory effect of Scl-AbIII or raloxifene on serum osteocalcin level was apparent following their administration for 4 weeks. Combined Scl-AbIII+raloxifene treatment was more effective than Scl-AbIII treatment alone in improving serum osteocalcin levels. This is in accordance with the study of Li et al. who reported significant increase of serum osteocalcin, recruitment and functional longevity of osteoblasts after Scl-Ab treatment.6

Humans with inherited sclerostin deficiency have increased bone mass and are resistant to fracture.19 However, the extent to which sclerostin might regulate bone formation and bone mass in a normal aged skeleton is not clear. The data of the current study describes the powerful anabolic response to sclerostin inhibition in OVX rats and clearly shows that sclerostin functions as a pivotal negative regulator of bone formation.

The evidence for an antiresorptive effect with Scl-Ab administration was observed in ovarietomy-induced bone loss rats6 and gonad-intact female monkey models.8 Alaee et al. showed enhanced bone repair following treatment of the rat femoral defect model with Scl-Ab.20 Furthermore, their results showed significantly 44% higher osteocalcin levels at 2 weeks of Scl-Ab treatment compared to the vehicle group. Tian demonstrated significant increase of bone mass by Scl-Ab treatment of immobilization/disuse rat model compared with their controls.21 Moreover, Sinder et al. reported that treatment of mouse model of osteogenesis imperfecta for 5 weeks with Scl-Ab had increased bone mass, cortical bone strength and induced bone formation on surfaces that are normally quiescent or resorptive in rapidly growing mice.22 Taken together, these findings suggested that antibody-mediated blockade of sclerostin represents a promising new therapeutic approach for the anabolic treatment of immobilization-induced osteopenia and a therapy during fracture healing to improve bone formation.23

Several reports suggest modulating effect of raloxifene on production of various factors involved in osteoclastogenesis and osteoclast survival, resulting in suppression of bone resorption.24 In this study, the treatment combined with Scl-AbIII and raloxifene significantly increased BSAP, osteocalcin and IGF-1 levels in comparison with Scl-AbIII treatment alone. Raloxifene is a unique agent that apparently possesses sufficient intrinsic activity to act like an agonist in bone and liver, but is a relatively pure antagonist in uterine tissue. In this study, raloxifene caused statistically significant increase in uterine weight in relation to the OVX controls. This effect may be related to the slight hypertrophy of the myometrium and endometrial stroma, which, in previous work, has been attributed to water retention.25

**CONCLUSION**

Combination therapy of Scl-AbIII and raloxifene can offer better gain over treatment with either one of them alone. These results support the potential of Scl-AbIII and raloxifene as attractive strategy to enhance bone formation. Confirmation of these preliminary observations must await careful long-term studies.

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