Study Of Pain-related Behavior And Immunohistochemical Analysis In The Hindlimb-unloaded Mice Model Of Bone Loss

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Introduction: Osteoporotic patients with no evidence of fractures sometimes experience vague lower back pain. A previous report has indicated that sensory innervation of ovariectomized rat vertebrae showed increased expression of the calcitonin gene-related peptide (CGRP), a neuropeptide marker of pain, and the transient receptor potential channel vanilloid subfamily member 1 (TRPV1), an acid-sensitive ion channel, in dorsal root ganglion (DRG) neurons1). However, there have been few reports regarding the correlation between osteoporosis and pain-related behavior, as well as between osteoporosis and immunohistochemical analysis. The objective of the current study was to investigate pain-related behavior and to elucidate the mechanism of osteoporotic pain by immunohistochemical analysis of the DRG in hindlimb-unloaded mice.

Methods: Male ddY mice (8 weeks old) were either hindlimb-unloaded (HU group) by tail suspension or hindlimb-loaded with only tail suspension (HL group = control group). The tail suspension lasted for 2 weeks. The femoral distal metaphyses and the proximal tibial metaphyses (HU group; 12 hindlimbs, control group; 8 hindlimbs) were analyzed three-dimensionally by micro-computed tomography (μCT) after the 2-week tail suspension. Mechanical sensitivity was also tested using von Frey filaments after the tail suspension. The frequency of the withdrawal response and the withdrawal threshold to the application of von Frey filaments to the plantar surface of the hindpaws was examined. To evaluate the frequency of the withdrawal response, three von Frey filaments with forces of 0.4, 0.6, 1.0, 1.4 and 2.0 g were applied 5 times each in ascending order of force, and the number and intensity of withdrawal responses were noted. Results were expressed as the percent response frequency of paw withdrawals. To evaluate the withdrawal threshold, each von Frey filament was applied once, starting with 0.008g and increasing until a withdrawal response was reached, which was considered a positive response. The lowest force producing a response was considered the withdrawal threshold. Immunohistochemical analysis of the CGRP expression and the TRPV1 expression was completed for the L4 and L5 DRG neurons. For both levels, there were 12 DRG from the HU group and 8 DRG from the control group. The ratio of CGRP-immunoreactive (ir) and TRPV1-ir cells to total DRG neurons was counted and averaged for each DRG neurons.

Results: μCT analysis of the distal femoral metaphysis and the proximal tibial metaphysis (Fig.1) showed that bone volume/tissue volume (BV/TV) and trabecular number (Tb.N) were significantly less in the HU group than in the control group, whereas trabecular separation (Tb.Sp) was significantly greater in the HU group than in the control group. The paw-withdrawal-frequency stimulated by von Frey filaments with strength of 0.4, 0.6 and 1.4 g were significantly higher in the HU group than in the control group.
Filaments of 1.0 and 2.0 g tended to be higher in the HU group than in the control group (Fig.2). The withdrawal threshold was significantly lower in the HU group than in the control group. The immunohistochemical analysis showed that CGRP expression was significantly higher in the HU group than in the control group in both L4 and L5 DRG neurons. TRPV1 expression was also significantly higher in the HU group than in the control group in both L4 and L5 DRG neurons (Fig.3).

**Discussion:** Skeletal unloading by spaceflight or prolonged bed rest causes a loss of bone in humans. The hindlimb-unloaded animal models are widely used in the study of bone loss, which are used as models of disuse osteoporosis. In this study, hindlimb-unloading induced significant bone loss in the hindlimbs of mice. The application of von Frey filaments to the planter surface of the hindpaw is often used to evaluate hindlimb hyperalgesia (e.g., knee joint inflammation, tibia fracture, etc.). In this study, mechanical hyperalgesia was observed in hindlimb-unloaded mice. CGRP has been reported to produce hyperalgesia via both Protein kinase A and C second-messenger pathways, thus elevated CGRP expression is suggested to produce pain. TRPV1 is a ligand-gated nonselective cation channel, which can be activated by capsaicin and other stimulation such as noxious heat and low pH. L Yu et al reported that TRPV1 in distinct subtypes of DRG neurons plays a role not only in the acute, but also in the chronic inflammatory pain, and that mechanical allodynia exists in chronic inflammatory pain, in which TRPV1 may also take effect. In this study, upregulation of CGRP and TRPV1 expression was recognized in DRG neurons innervating hindlimbs. The hindlimb-unloading leading to bone loss may induce upregulation of CGRP and TRPV1 expression in DRG neurons innervating the hindlimbs.

**Significance:** In this study, hindlimb-unloading by tail suspension induced bone loss and mechanical hyperalgesia in hindlimbs. The hindlimb-unloading also induced upregulation of CGRP and TRPV1 expression in DRG neurons innervating the hindlimbs. The results suggest that hindlimb-unloaded mice provides a useful model for osteoporotic pain.

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**Fig. 1 : μCT analysis**

- femoral distal metaphysis
- proximal tibial metaphysis

![μCT analysis images](image-url)
Fig. 2: pain related behavior

Paw Withdrawal Frequency Stimulation

HU group: hindlimb-unloading
control group (HL group): hindlimb-loading

<table>
<thead>
<tr>
<th>Force (g)</th>
<th>HU</th>
<th>Control</th>
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<tr>
<td>0.4</td>
<td>60</td>
<td>30</td>
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<tr>
<td>0.6</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>1.0</td>
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<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>2.0</td>
<td>20</td>
<td>10</td>
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P = 0.031, P = 0.001, n.s., P = 0.039, n.s.

Fig. 3: Immunohistochemical analysis

CGRP expression in DRG

L4

HU: 45%, Control: 10%
P = 0.0001

L5

HU: 40%, Control: 10%
P = 0.0001

TRPV1 expression in DRG

L4

HU: 40%, Control: 10%
P = 0.001

L5

HU: 40%, Control: 10%
P = 0.0001

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