SHORT COMMUNICATION

Hindlimb Suspension Does Not Influence Mechanical Sensitivity, Epidermal Thickness, and Peripheral Nerve Density in the Glabrous Skin of the Rat Hind Paw

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Summary
Our aim was to investigate the influence of microgravity on the sensitivity of the skin to mechanical stimulation, epidermal thickness, peripheral nerve density in the upper dermis, and serum levels of a stress marker in a rat hindlimb suspension (HS) model. Thirty 8-week-old male Wistar rats were randomly divided into 3 groups: HS, n=10; sham HS, n=10; control, n=10. The suspension system was attached to rat tails in both the HS and sham-HS groups, but the hindlimbs were suspended only in the HS group. The HS and sham-HS groups were treated for 4 weeks. In behavioral tests using von-Frey filaments (n=5 in each group), mechanical hypersensitivity developed in the HS group. Serum corticosterone levels increased significantly in the HS and sham HS groups compared to the control group, and no changes in epidermal thickness or peripheral nerve density were observed immediately after the removal of HS (n=5 in each group). These data indicated that the mechanical hypersensitivity observed in the HS group was not caused by microgravity or inactivity, but rather by restraint stress. We suggest that microgravity does not affect skin sensitivity and histology in these animals.

Key words
Hindlimb suspension • Mechanical hypersensitivity • Epidermal thickness • Peripheral nerve • Stress

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Hindlimb suspension (HS) animal models are widely used in studies investigating the effect of microgravity and spaceflight on the locomotorium such as bone and muscle (Templeton et al. 1984). Changes in the viscera (Stein et al. 2005), circulation (De Salvatore et al. 2004), and blood vessels (Fujino et al. 2005) have also been studied using the HS model. A few histochemical studies that investigated muscular sensation in response to HS showed no effect on muscle spindles or the size and shape of Golgi tendon organs (Asmussen and Soukup 1991). However, extremely few studies have addressed the changes in skin tissues and peripheral nerves or the sensory aspects of suspension hypokinesia. Changes in skin tissue and peripheral nerves may affect the skin sensation (Li et al. 2009, Nakano et al. 2012). Although a few reports showed that microgravity and non-weight bearing are associated with pressure hypersensitivity in rats, the effect of stress was not examined using a sham group in these studies.
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In studies using HS model rats, the influence of restraint

stress should not be ignored because HS affects the

wellness of the animal. A previous study showed

increased serum levels of stress markers such as

epinephrine in HS model rats (Kawano et al. 1995). Thus,

whether hypersensitvity is a specific consequence of

microgravity, or if other stimuli can also cause it, remains

unclear. To address these issues, we examined the

changes in the sensitivity of the skin in response to

mechanical stimuli, epidermal thickness, peripheral nerve
density in the upper dermis, and serum levels of a stress
marker by using HS model rats.

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University. Thirty 8-week-old male Wistar rats weighing

220±18 g from Kudo Laboratories (Tokyo, Japan) were

randomly divided into control, HS, and sham-HS groups

(n=10 each). Rats were housed individually in 30 × 40 ×

20 cm cages at 24 °C, with water and food available ad

libitum. The suspension system was attached to the tail of

rats in the HS group, and the height of the system was

adjusted so that the suspended hindlimbs did not touch

the floor (Templeton et al. 1984). The same suspension

system was attached to the tails of rats in the sham-HS

group but the hindlimbs were not suspended, so the rats

could move normally in their cages. All other aspects of

the set-up in the three groups were identical. The period

of HS was 4 weeks.

Behavioral tests to assess bilateral hindlimb

mechanical sensitivity were performed weekly in 5 rats

each group. Rats were placed in a small animal

fixator in a quiet room during the behavioral test. The

glabrous skin of the hind paw was probed 10 times with a

von Frey filament (15 g; North Coast Medical, USA)

repeatedly, and the paw-withdrawal response (PWR) was

determined. The body weights of rats in the 3 groups

were also measured weekly. At the end of the HS period,

the suspension system was removed in the HS and sham-

HS groups. The rats that underwent behavioral testing

were maintained in a normal breeding state, and the

behavioral tests and weight measurements were extended

for 8 weeks after the removal of HS. The other 5 rats in

each group that were not subjected to behavioral tests

were intraperitoneally anesthetized with sodium

pentobarbital (40 µg/g) immediately after the removal of

HS, and blood was collected by venipuncture of the tail.

The serum was extracted after clotting and centrifugation

of blood, and it was stored in the freezer. Subsequently,

transcardial perfusion was performed with 4 %

paraformaldehyde in 0.1 M phosphate buffer, and the

glabrous skin of the hind paw was removed bilaterally.

The tissues were embedded in tragacanth gum after

soaking in 10 % saccharose and stored at −80 °C. Serum
corticosterone levels were estimated as a stress marker

using the Corticosterone EIA kit (YK240; Yanaihara

Institute Inc., Japan) (Hong et al. 2011). Although the

influence of pentobarbital anesthesia cannot not be

ignored in the determination of serum corticosterone

levels (Green and Moor 1977), its effect on the results

was minimized by employing uniform anesthesia

conditions for all rats during blood sampling. Frozen skin

sections (10 µm) were cut using a cryostat (CM1950;

Leica, Germany) and stained with hematoxylin-eosin.

Epidermal thickness was measured using the Scion Image

software (Scion Corp., USA) at 7 random points per

image (×200). Five sections per skin sample were used

for measurements. Immunohistochemical staining for

peripheral nerves was performed on frozen sections

(30 µm) with rabbit anti-PGP9.5 antibody (1:2000;

Calbiochem, Germany), as previously described (Lauria

et al. 2005). For semiquantitative analysis, the number of

PGP9.5-positive nerve fiber fragments in the upper
dermis (0-50 µm in depth under the epidermis) was

counted on the images (×200) obtained from 5 sections

per skin sample and classified according to the skin tissue

width. The number of PGP9.5 nerve fibers per 1 mm-unit

was calculated as “peripheral nerve profiles.” Data are

presented as mean ± SEM. Statistical analysis was

performed using ANOVA with the Scheffe post-hoc test,

and P<0.05 was considered significant.

Although body weight increased at all time

points in the HS, sham-HS, and control groups, weight

gain in the HS and sham-HS groups was lower than that

in the control group (Fig. 1A). The PWR increased

significantly during HS in the HS and sham-HS groups

compared to the control group (P<0.01), and this increase

in the PWR persisted for 5 and 6 weeks after removal of

HS (Fig. 1B). However, no significant differences in

body weight and PWR were observed between the HS

and sham-HS groups. Serum corticosterone levels

increased significantly in the HS and sham-HS groups

compared to the control group (P<0.01) (Fig. 2A), with

no significant differences in corticosterone levels

between these 2 groups. In the histological analysis, no

pathological changes such as inflammation were
observed in skin tissue. No significant changes in epidermal thickness or peripheral nerve profiles in the glabrous skin of the hind paw were observed in any of the groups (Fig. 2B,C).

![Graph A](image1.png)

**Fig. 1.** Changes in body weight (A) and mechanical sensitivity (B) during and after HS. The HS and sham-HS procedures resulted in a significant decrease in weight gain; no significant difference was observed between the HS and sham-HS groups (A). The PWR induced by 15-g von Frey filaments significantly increased in the HS and sham-HS groups during HS compared to the control group; no significant difference was observed between the HS and sham-HS groups (B). HS: hindlimb suspension. * vs. control group, P<0.05.

![Graph B](image2.png)

**Fig. 2.** Changes in serum corticosterone levels (A), epidermal thickness (B), and peripheral nerve density in the upper dermis (C) immediately after the removal of HS. Serum corticosterone levels significantly increased in the HS and sham-HS groups compared to the control group; no significant difference was observed between the HS and sham-HS groups. The procedures performed in the HS and sham-HS groups did not cause changes in epidermal thickness and density of PGP9.5-positive nerve fibers in the upper dermis. * vs. control group, P<0.05.

No differences were observed between the HS and sham-HS groups in any of the parameters tested. Chowdhury et al. (2011) suggested that non-weight bearing conditions in HS model rats may lead to pressure hypersensitivity. In the present study, although the same HS model was used, mechanical hypersensitivity was observed in the sham-HS group as well. Rats in the sham-HS group were attached to the trolley suspension system in the same manner as those in the HS group during HS, and they were allowed to bear weight on their hindlimbs while walking. Our results indicate that the mechanical hypersensitivity observed during HS cannot be attributed to the effects of microgravity. In addition, changes in epidermal thickness and peripheral nerve density, which are often observed in diabetes and neuropathy (Chao et al. 2011, Kojundzic et al. 2008, Lauria et al. 2005, Romanovsky et al. 2010), were not detected in the HS group. These results indicate that the hypersensitivity in the HS group was not due to histological changes of peripheral tissues such as skin and nerve. Because a
similar increase in the serum levels of corticosterone, which was used as a stress marker, was observed in the HS and sham groups, it is possible that mechanical hypersensitivity in these groups was induced by stress. Body weight analysis confirmed the involvement of stress. Attachment of the suspension system may have resulted in the development of restraint stress and possibly mechanical hypersensitivity (Bardin et al. 2009, Imbe et al. 2004, Nasu et al. 2009).

Inactivity and immobilization of extremities, such as those associated with casting after fracture, have been reported to induce hypersensitivity (Allen et al. 1999, Guo et al. 2004). Terkelsen et al. (2008) conducted an experiment involving healthy human subjects in which the forearm was immobilized by casting for 4 weeks; subsequently, transient movement-provoked pain and increased skin temperature in concert with mechanical and cold hyperalgesia were induced in distal parts of the immobilized extremity. Although the detailed mechanisms are unclear, several factors have been associated with the induction of hypersensitivity, including hypokinesia; reduction of the sensory input; changes in peripheral tissue, including the skin; blood flow deficiencies; central neuron system changes; and stress. In our HS model, and considering the results of the study, hypokinesia, reduction of sensory input, and changes in the skin tissue can be excluded. However, stress may have affected the results of various parameters, and further investigation will be necessary in the future.

In conclusion, mechanical hypersensitivity observed in a HS model rat was related to stress but not to microgravity. In addition, our results indicated that microgravity did not affect the sensitivity or histology of the glabrous skin.

Conflict of Interest
There is no conflict of interest.

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References


