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ORIGINAL ARTICLE

# Involvement of irisin in passive blood flow restriction for preventing disuse muscle atrophy in rats

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## ABSTRACT

The purpose of this study was to determine the effect of blood flow restriction (BFR) without exercise on muscle fibre atrophy by muscle wet weight and muscle relative weight ratio. Furthermore, to confirm the involvement of irisin in BFR by measuring the haemodynamics of irisin, which has an inhibitory effect on muscle atrophy after BFR. Male Wistar rats were assigned to three groups: spontaneous rearing group, tail suspension group and tail suspension plus blood flow restriction group. Soleus muscle wet weight, relative weight ratio, and serum concentrations of irisin were determined after the respective treatments for 6 weeks. The muscle relative weight ratio of the soleus were greater in the tail suspension plus BFR group compared to the tail suspension group ($p < .05$). Significantly lower serum irisin levels in the tail suspension plus BFR group versus the natural rearing group ($p < .05$). The effect of blood flow restriction without exercise in preventing the progression of muscle atrophy was confirmed by assessment of muscle dissection. Blood sampling immediately after BFR significantly reduced serum irisin levels in rats whose muscle atrophy was suppressed by BFR, relative to the control group.

**Keywords**: Sport medicine, ELISA, Muscle wet weight, Muscle relative weight ratio.

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## INTRODUCTION

Skeletal muscle atrophy occurs in a short period of time when skeletal muscle is inactive, such as in the case of bed rest or joint immobilization. Muscle strength decline in patients on bed rest occurs at a rate of about 1-3%/day, or 10-15%/week and declines to about 50% in 3 to 5 weeks (Ishikawa and Tsuji,2016). Therefore, preventing the progression of disuse muscle atrophy is an important issue.

Temporary passive blood flow restriction (BFR) without exercise has been reported to prevent muscle atrophy. Takarada et al. (2000) studied muscle atrophy in the peri-knee muscles of patients after anterior cruciate ligament reconstruction surgery. Two groups of patients were examined: a group on a regular exercise therapy program and a group that received additional BFR. They reported an effective reduction in disuse atrophy of the extensor muscles of the knee in the group with added BFR compared to the group that received the usual exercise therapy program. Kubota et al. (2008) immobilized each of the healthy men's left ankles using a cast, and participants were instructed to walk with non-weight bearing using crutches during this period. They were then randomly divided into three groups: a group with regular BFR, a group that received isometric strength training, and a group that did nothing. After two weeks, muscle strength and thigh circumference were examined. The results showed a smaller decrease in muscle strength and thigh circumference in the group with regular BFR than in the other groups.

In long-term human evaluations, prevention of progression of muscle atrophy by temporary BFR without exercise has been assessed by muscle strength, muscle circumference and muscle cross-sectional area (Takarada et al.,2000, Kubota et al.,2008, Kubota et al.,2011), but not by muscle dissection. In animal studies, muscle dissection allows quantification.

In recent years, a myokine called irisin has been discovered and has attracted attention. Irisin is produced by the skeletal muscle after exercise (Boström et al.,2012). It has been reported that administration of irisin to mice suppressed muscle and bone atrophy induced by disuse due to non-weight bearing (Colaianni et al.,2016). It has also been shown from the anterior tibialis muscle that BFR to the proximal thigh enhances the expression of fibronectin type III domain-containing 5 protein, which is thought to be the origin of irisin (Nakajima et al.,2018). Nevertheless, the secretory response of irisin to passive BFR without exercise is not clear.

The purpose of this study is to determine the effect of blood flow restriction without exercise on muscle fibre atrophy by means of muscle wet weight and muscle relative weight ratios. A further step is to confirm the involvement of irisin in blood flow limitation by measuring serum concentrations of irisin.

## MATERIAL AND METHODS

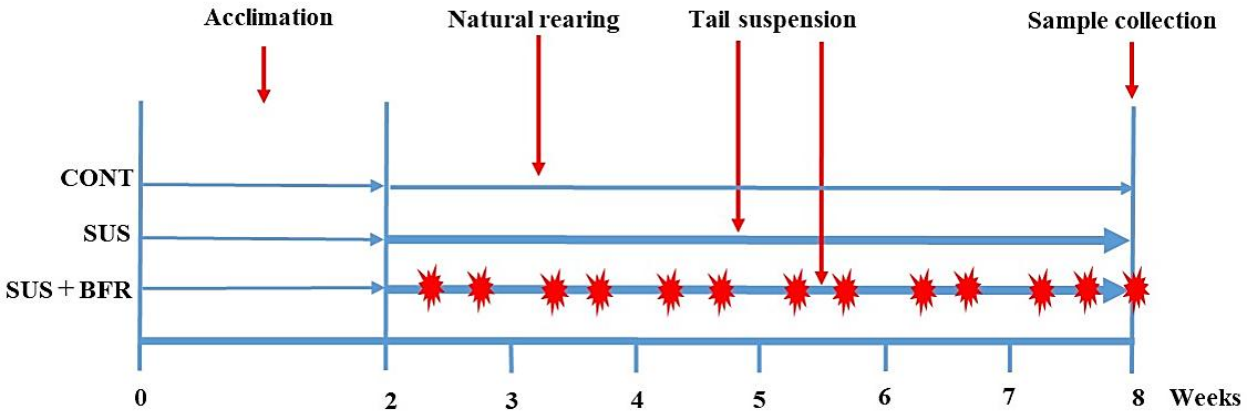
### *Ethical considerations*

This study was approved by the Kibi International University Research Ethics Review Committee (Approval No. A18-04).

### *Animals and experimental protocol*

Twenty-one 12-week-old male Wistar rats were used in this experiment. The rats were brought in at 10 weeks of age, and after two weeks of acclimatization, they were subjected to the experiment. Rats were randomly divided into three groups: tail suspension (SUS), temporary passive BFR load (SUS + BFR), and natural rearing (control group, CONT) (n = 7 per group). Rats in the SUS and SUS + BFR groups were subjected to

tail suspension to induce disuse muscle atrophy. The animal rearing room was maintained at 23 ± 1°C and kept on a 12:12 h light/dark cycle. The rats were given free access to food (Clare CE-2) and water. Six weeks later, all groups were euthanised by intraperitoneal overdose with sodium pentobarbital, followed by collection of the left soleus muscle and blood samples from the heart for anatomical and biochemical assessment (Figure 1).




*Note. n = 7 for each group. CONT; control group. SUS; tail suspension group. SUS + BFR; tail suspension plus temporary passive blood flow restriction group. Blood flow restriction.*

Figure1. Experimental protocol.

***Induction of muscle atrophy of the lower limb muscles (tail suspension)***

In the present study, the tail suspension technique was used to induce muscle atrophy in the lower limb muscles. The tail suspension was applied with a modification of the method described by Morey et al (Morey-Holton et al.,1981, Ohmori et al.,1997, Harada et al.,2005). An octopus thread string was secured to the tail with adhesive tape and the tail was suspended from a guide rail passed over the gauge ceiling so that the sole of the foot did not touch the floor. The rats could move around the cage using their grounded upper limbs. They were free to eat food and drink water (Figure 2).

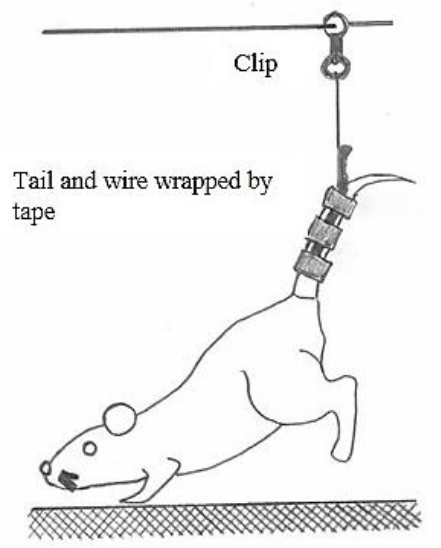



Figure 2. The tail-suspension rat model.

The height of the guide rail was adjusted to minimize the stress on the rat due to suspension so that the sole of the rat's feet did not touch the bottom of the cage. The clip that connects the octopus thread to the guide rail can be rotated 360° and moved by sliding along the guide rail so that it can be moved effortlessly with a grounded upper limb.

### *Blood flow restriction*

The rats were once taken down from tail suspension and placed in the animal folder under anaesthesia-free conditions with upper body immobilisation, foot immobilisation and knee joint extension, and a manchette was wrapped around the left lower limb to restrict blood flow. A manchette for premature infants (25 mm wide and 90 mm long; Nakamura Medical Industry Co., Ltd., Tokyo, Japan) was used for BFR (Imano et al.,2020). The BFR loading condition was a cuff pressure of 300 mmHg (Tanimoto and Ishii,2006) held for 6 minutes (Hamaoka et al.,1994), and the frequency and duration of the experiment were twice a week for 6 weeks (Sudo et al.,2017). Blood flow restriction was also performed immediately before euthanasia in order to measure serum irisin concentrations immediately after the end of blood flow restriction.

### *Tissue measurement*

The wet weight of the excised left soleus muscle was measured. The blood samples were centrifuged at 4000 rpm for 10 minutes at 4°C. Serum concentration of irisin was measured using an enzyme-linked immunosorbent assay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, Cat. No. EK-067-29). Serum concentrations of irisin were calculated using a microplate reader SH-1200 (Corona Electric Co., Ltd., Ibaraki, Japan) and curve-fitting software SF-6 with a four-factor logistic function. The mean value of triplicated wells was used for analysis.

### *Statistical analysis*

Assumptions of normality were checked using the Shapiro-Wilk test for data on muscle wet weight, muscle relative weight ratio and serum irisin concentration. For data on muscle wet weight and relative weight ratio, one-way analysis of variance was used to examine the main effects between groups for each outcome. Following that, the Tukey's method was performed as a post-hoc test. For data on serum irisin concentrations, the Kruskal-Wallis test was used to compare differences across the three groups. For the post-hoc tests, the Dann-Bonferroni method was used to adjust the value of the probability of significance for the two-group test. Statistical analysis was performed using IBM SPSS Statistics for Windows version 26 (IBM Corp. Armonk. NY. USA). The level for statistical significance was set at $\alpha = .05$. Values are presented as mean ± standard error.

## RESULT

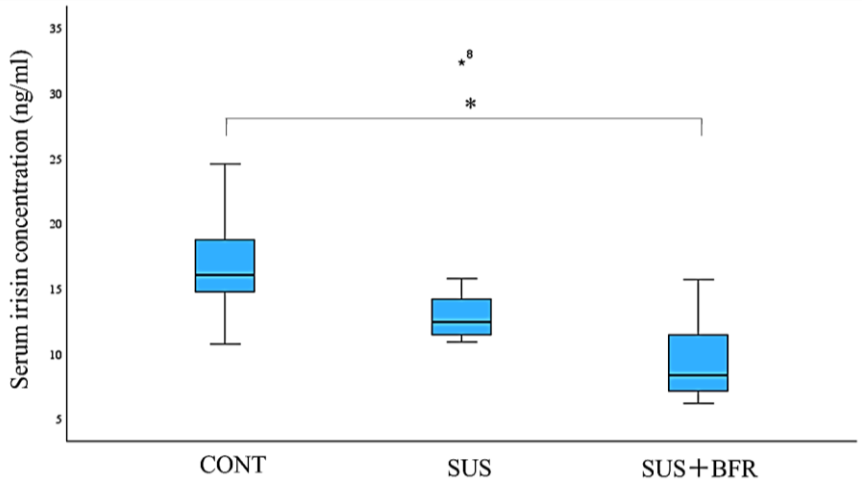
Table 1 shows a comparison of soleus muscle wet weight and muscle relative weight ratios for each group. In terms of muscle wet weight, both the SUS and SUS+BFR groups showed significantly smaller values than the CONT group. No significant differences were found between the SUS and SUS+BFR groups. In terms of relative weight ratio, the SUS+BFR group showed significantly greater values than the SUS group ($p < .05$) (Table1).

Figure 3 shows a comparison of serum irisin concentrations in each group. Significantly lower serum irisin levels in the SUS + BFR group (9.67 ± 1.29 ng/ml) versus the CONT group(16.95 ± 1.65ng/ml) ($p < .05$). There was no significant difference between the CONT and SUS groups(15.32 ± 2.91ng/ml) (Figure 3).

Table1. Comparison of muscle wet weight, relative weight ratio of each group (mean ± standard error).

| Group | n | MW (mg) | MW/BW (mg/g) | MW/BW 95% Confidence Interval |
|---|---|---|---|---|
| | | | | Lower – Upper |
| SUS | 7 | 69.0 ± 3.7† | 0.17 ± 0.01 † | 0.14 – 0.19 |
| SUS + BFR | 7 | 82.0 ± 6.7† | 0.22 ± 0.02*† | 0.17 – 0.27 |
| CONT | 7 | 182.7 ± 6.5 | 0.39 ± 0.01* | 0.37 – 0.42 |

*Note. *: p < .05 (vs SUS). †: p < .05 (vs CONT). Statistical analysis: Tukey's method. BW; body weight. MW; muscle wet weight.MW/BW; muscle relative weight ratio. CONT; control group. SUS; tail suspension group. SUS + BFR; tail suspension plus temporary passive blood flow restriction group.*




*Note. *p < .05 (Dunn-Bonferroni post hoc method). n = 7 for each group. CONT; control group. SUS; tail suspension group. SUS + BFR; tail suspension plus blood flow restriction group.*

Figure 3. Comparison of serum irisin concentrations in each group (mean ± standard error).

## DISCUSSIONS

The muscle relative weight ratio of the soleus were greater in the tail suspension plus blood flow restriction group compared to the tail suspension group ($p$ < .05). These results suggest a preventive effect of BFR without exercise on the progression of muscle atrophy.

It is reported that restricting blood flow to the muscle by generating sustained muscle tonic force through slow movement can increase the muscle hypertrophy effect even at low intensity (Tanimoto and Ishii,2006). It has been identified that there is a reduction in tissue oxygen saturation ($StO_2$) in muscles due to this movement, which is speculated to be a factor in muscle hypertrophy. In response to this, we hypothesize that the hypertrophic effects of BFR-only conditions without exercise may also be due to reduced oxygen saturation. Near-infrared spectroscopy has confirmed that $StO_2$ in rat lower limb muscles is reduced in BFR using the manchette in this study, creating a hypoxic environment within the muscle (Imano et al.,2020).

Muscle protein synthesis is enhanced by various stimuli, including mechanical stimuli and growth factors. The regulation of this process is controlled by the mammalian target of rapamycin (mTOR) signalling system. The mTOR signalling system facilitates muscle protein synthesis by promoting the translation of mRNA at the ribosome, a cellular organelle. BFR without exercise is thought to be a novel stimulus to prevent disuse muscle atrophy because of its ability to promote muscle protein synthesis signalling. Sudo et al. (2017)

showed hypertrophy of the muscle fibre cross-sectional area in the tibialis anterior muscle of rats with BFR without exercise. Nakajima et al. (2016) reported in rats that BFR without exercise significantly reduced microvascular $O_2$ partial pressure and significantly increased phosphorylation of p70 S6-kinase and ribosomal protein S6, downstream targets of mTOR.

In an experiment examining the relationship between exercise intensity and blood irisin concentration, it was reported that serum irisin concentration immediately after exercise decreased significantly compared to that before exercise during low-intensity exercise (40% $VO_2$max, treadmill running for 40 min) and then continued to increase (Tsuchiya et al.,2014). Unexpectedly, the significant decrease in serum irisin concentrations in this study could be attributed to the fact that blood was drawn immediately after the BFR. In addition to skeletal muscle, other cells that secrete irisin include cardiac muscle, adipose tissue, pancreas, liver, and brain (Aydin et al.,2014, Roca-Rivada et al.,2013). The mechanism of the rapid decrease immediately after exercise is unknown, but it has been partially explained by the presence of multiple sources of irisin production and their interaction with other cytokines and hormones (Tsuchiya et al.,2014).

This study had some limitations. The cuff pressure in this study may be too high for adaptation to humans. Kubota et al (2011) reported that the application of BFR without exercise to the lower limb (50 mmHg, 5 minutes, 5 sets, twice a day, for 14 days) prevented disuse muscle weakness caused by weightless immobilisation. Second, the hind limb was grounded because the Tail suspension was removed when performing BFR. In addition, some rats' hindlimbs landed on the floor during the suspension period. In the SUS group, one rat landed twice, and one rat landed three times. In the SUS + BFR group, three rats landed once, two rats landed twice, and one rat's hindlimb landed three times. Future research prospects include a longitudinal study of irisin blood levels under BFR without exercise for disuse muscle atrophy.

## CONCLUSIONS

Muscle dissection confirmed that restriction of blood flow without exercise prevented the progression of muscle atrophy. Blood sampling immediately after BFR significantly reduced serum irisin levels in rats whose muscle atrophy was suppressed by BFR, relative to the CONT group.

## AUTHOR CONTRIBUTIONS

Tetsuo Imano: study design, data analysis, manuscript preparation. Masaaki Nakajima: study design, data analysis.

## SUPPORTING AGENCIES

No funding agencies were reported by the author.

## DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author.

## ETHICS STATEMENT

The experiments completed in this study comply with the current laws of the country in which they were performed.

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