



Research article

Low-frequency vibration promotes AMPK-mediated glucose uptake in 3T3-L1 adipocytes

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HIGHLIGHTS

- Low-frequency vibration (LFV) of 50 Hz at 1000–1500 mVpp over 5 days promotes glucose uptake in 3T3-L1 adipocytes.
- LFV does not promote insulin-mediated GLUT4 translocation but AMPK-mediated GLUT4 translocation into the plasma membrane.
- LFV has the potential to activate mechanosensors, which promote AMPK-mediated GLUT4 translocation and glucose uptake.
- AMPK-mediated glucose uptake induced by LFV in adipocytes may potentially improve insulin resistance in diabetes mellitus.

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ABSTRACT

Delayed healing of diabetic foot ulcers (DFUs) is one of the major consequences of angiopathy caused by hyperglycemia stemming from insulin resistance. Interventions that improve blood supply and hyperglycemia are essential for treating DFUs. Low-frequency vibration (LFV) promotes peripheral blood flow and wound healing in DFUs, regardless of hyperglycemia. We hypothesized that LFV promotes non-insulin-mediated glucose uptake, which is also referred to as AMPK-mediated glucose uptake, in adipocytes at wound sites, thereby alleviating hyperglycemia, which, in turn, accelerates wound healing. The objective of this *in vitro* study was to identify LFVs that optimally promote glucose uptake in adipocytes and investigate the mechanism underlying enhanced glucose uptake caused by LFV. 3T3-L1 adipocytes were used in this study. LFV was applied at 50 Hz for 40 min/d to investigate the most effective vibration intensity (0–2000 mVpp) and duration (0–7 d) of glucose uptake. We comparatively assessed 2-deoxyglucose (2-DG) uptake in control and vibration groups. To elucidate the mechanism underlying 2-DG uptake induced by LFV, wortmannin and compound C were used to inhibit insulin-mediated GLUT4 translocation and AMPK activation, respectively. Additionally, GLUT4 translocation to the plasma membrane was assessed using immunofluorescence image analysis. Our results indicated that 2-DG uptake in the 1000 and 1500 mVpp groups was higher than that in the control group ($p = 0.0372$ and 0.0018 , respectively). At 1000 mVpp, 2-DG uptake in the 5- and 7-d groups was higher than that in the non-vibration group ($p = 0.0169$ and 0.0452 , respectively). Although wortmannin did not inhibit 2-DG uptake, compound C did. GLUT4 translocation to the plasma membrane was not observed in the vibration group adipocytes treated with compound C. Thus, our results indicated that an LFV of 50 Hz, 1000 mVpp, 40 min/d, over 5 d was optimal for accelerating AMPK-mediated GLUT4 translocation and glucose uptake in adipocytes.

1. Introduction

Diabetic foot ulcers (DFUs) are hard-to-heal wounds that may result in lower extremity amputations and deaths because of infection [1]. As the global prevalence of DFUs rises, finding effective treatments for DFUs

has become imperative. Improving hypoglycemia and insulin resistance for underlying diabetes mellitus are crucial in treating DFUs [2, 3]. Additionally, interventions that improve the blood supply and promote blood flow to lower extremities are essential for treating DFUs [4]. Conventional methods that promote blood flow [5, 6, 7] are invasive and

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carry the risk of infection. Furthermore, some patients are not eligible for surgery. Therefore, we focused on low-frequency vibration (LFV) therapy, which is a non-invasive method that promotes blood flow. Generally, LFV accelerates regional blood flow via vasodilation by producing nitric oxide (NO) [8, 9] and causes wound healing in pressure injuries [10, 11]. Hyperglycemia inhibits NO production by both blocking endothelial NO synthase activation and promoting dysfunctional arginine production via the polyol pathway [2], however, LFV promotes wound healing of DFUs [12]. Thus, we hypothesized that LFV improves local insulin resistance at wound sites by increasing glucose uptake. LFV-mediated improvement of local insulin resistance can inhibit the polyol pathway, which in turn promotes arginine production. As arginine is a substrate of NO synthase, increased arginine production stimulates NO synthesis, which then promotes blood flow via vasodilation, thereby accelerating the healing of wounds associated with DFUs.

The glucose transporter, GLUT4, is a major regulator of blood glucose levels. GLUT4 translocation to the plasma membrane is categorized into two types: insulin-mediated translocation and AMP-activated protein kinase (AMPK)-mediated translocation [13]. Reportedly, whole-body vibration has been used to induce AMPK-mediated GLUT4 translocation in skeletal muscles [14]. However, DFUs may occur around metatarsals and heels, where muscles are scarce or atrophied due to peripheral neuropathy. Therefore, in this study, we focused on glucose uptake in adipocytes. Previous studies have indicated that the AMPK activator, AICAR, accelerates GLUT4 translocation in adipocytes [15]. Thus, we hypothesized that LFV may promote AMPK-mediated GLUT4 translocation in adipocytes.

The purpose of this study was to explore optimal vibratory conditions, including vibration intensity and the duration, required to promote glucose uptake in adipocytes. Furthermore, we aimed to reveal the mechanisms underlying the promotion of glucose uptake in adipocytes by LFV via inhibition of the insulin signal pathway and AMPK activation.

2. Materials and methods

2.1. Reagents

Insulin (human, recombinant), dexamethasone, 2-deoxyglucose (2-DG), and wortmannin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The details of the other critical reagents are as follows: dorsomorphin (compound C) from Abcam (Cambridge, UK); oil red O from Cosmo Bio (Tokyo, Japan); primary antibodies against GLUT4 (rabbit, polyclonal, #NBP1-49533) from Novus Biologicals (Littleton, CO); and fluorescent antibody Alexa Fluor® 488 (donkey, polyclonal, #711-545-152) from Jackson ImmunoResearch (West Grove, PA). Details regarding reagents have been placed in the supplemental materials.

2.2. Cell culture

3T3-L1 cells were obtained from the Japanese Collection of Research Resources Cell Bank (Osaka, Japan). 3T3-L1 cells were seeded in a six-well culture plate at a density of 3×10^4 cells/mL and cultured in DMEM with low glucose supplemented with 10% (v/v) heat-inactivated FBS and 5% (v/v) penicillin-streptomycin solution until confluence was reached (day 0). Post-confluence, cells were cultured for 3 d in DMEM with low glucose containing 10% FBS, 10 μ g/mL insulin, 1 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Thereafter, the cells were cultured in DMEM with low glucose containing 10% FBS and 10 μ g/mL insulin. Three days later (day 6), cells were cultured in DMEM with high glucose containing 10% FBS for 2 d. 3T3-L1 adipocytes that were 8–14 d past initial differentiation were used for the experiments.

2.3. Vibration profiles and experimental set-up

A previously developed vibrator was used to apply vibrations [8, 9, 11]. While using this vibrator, changes in the intensity of vibration were

achieved by changing the voltage of the power supply. The vibrator was mounted on a triaxially mobile manipulator. The six-well plate was placed on the oscillator and rubber foam mat and fixed with bands. Vibration was applied to the cells in the incubator at 37 °C, and vibration could be transmitted as mechanical stimuli to the cells in the medium through six-well plate.

Vibration intensities of 600, 800, 1000, 1500, and 2000 mVpp were applied to 3T3-L1 adipocytes at a fixed frequency of 50 Hz for 40 min/d for 7 d starting from day 8, in order to verify whether vibration induces the promotion of glucose uptake in adipocytes at day 14, as well as to determine the specific vibration intensity needed to promote such glucose uptake. Next, vibration was applied at 50 Hz as well as at the most effective vibration intensity, for 40 min/d to investigate the duration of the most effective vibration required. 3T3-L1 adipocytes that were 14 d past initial differentiation but not subjected to vibration treatment were used as the control group.

2.4. 2-DG uptake test

A 2-DG Uptake Measurement Kit (Cosmo Bio) was used to perform the 2-DG uptake test on 3T3-L1 adipocytes at day 14 in the treatment and control groups. The 3T3-L1 adipocytes were pre-incubated at 37 °C in serum-free DMEM with high glucose for 6 h. After pre-incubation, cells were incubated for 30 min with 2 mL of Krebs-Ringer phosphate-HEPES buffer (KRPH; 50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM CaCl₂, and 1.3 mM MgSO₄) containing 2% bovine serum albumin (BSA). Next, insulin (1 μ M) was added to the KRPH buffer, and the cells in both groups were incubated for 20 min, following which both vibration and insulin treatments were simultaneously applied to the vibration group. In contrast, the control group only received insulin treatment. Cells subjected to insulin treatment were used as a positive control, and we investigated the effect of LFV on 2-DG uptake in the presence and absence of insulin in adipocytes. Following insulin treatment, 1 mM 2-DG was applied and cells were incubated for 20 min. Next, cells were washed thrice with cold phosphate-buffered saline (PBS). Washed cells were lysed using 1 mL Tris/HCl buffer (10 mM, pH 8.0). Cell lysate was collected in a tube and heated to 80 °C for 15 min. Subsequently, the preparation was centrifuged at $15,000 \times g$ for 20 min at 4 °C, then the supernatant was transferred to a new tube. 2-DG uptake was quantified via a linear plot of luminescence signal vs. concentration of authentic 2-DG6P (μ mol/well).

Insulin-mediated GLUT4 translocation and AMPK activation inhibitors were used to verify whether vibration increases 2-DG uptake via insulin-mediated glucose uptake or AMPK-mediated glucose uptake. In order to inhibit glucose uptake signal pathways, 3T3-L1 adipocytes were pre-incubated with either 100 nM wortmannin (an insulin-mediated glucose uptake inhibitor) for 30 min or 20 μ M compound C (an AMPK activation inhibitor) for 16 h, before vibration treatment, and same treatments were performed in the non-vibration group to investigate the effectiveness of those compound in the non-vibration group. DMSO was used as vehicle control (final concentration was 0.1%). The amount of 2-DG6P incorporated into cells was determined using the 2-DG Uptake Measurement Kit and 2-DG uptake was quantified.

2.5. Adipogenesis assay using oil red O staining

Oil red O staining, which is used to determine adipocyte differentiation, stains the lipid droplets within cells, whereupon the amount of extracted dye is measured in order to determine the volume of accumulated lipid. Treated adipocytes were fixed in 10% formalin, following which intracellular lipid droplets were stained using oil red O solution (Cosmo Bio). Following microscopic observation using an inverted microscope (BZ-X710, Keyence, Osaka, Japan), the absorbance of dye extraction at 540 nm was measured using a spectrophotometer (SpectraMax iD3, Molecular Devices, San Jose, CA). Staining and the dye

extraction assay were conducted according to the manufacturer's instructions.

2.6. Lactate dehydrogenase (LDH) cytotoxicity assay

The LDH cytotoxicity assay provides a colorimetric format for measuring and monitoring cell cytotoxicity. After treating adipocytes at day 14 with wortmannin or compound C, an LDH assay was performed using a CytoSelect™ LDH Cytotoxicity Assay Kit from Cell Biolabs, Inc. (San Diego, CA), following the manufacturer's instructions. After applying the reagent, the plate was incubated at 37 °C for 0.5 h and OD was measured at 450 nm as the primary wavelength. LDH activity released in the non-vibration and each group treated with inhibitors was expressed as a percent of the maximum LDH released in lysed nontreated cells (% cytotoxicity).

2.7. Immunofluorescence of GLUT4

3T3L-L1 adipocytes in the non-vibration and vibration groups were fixed with 10% formalin for 30 min. Subsequently, formalin was removed and cells were washed thrice with PBS for 5 min. To block nonspecific antibody binding, cells were incubated in 1% BSA/PBS for 30 min at room temperature. Cells were incubated overnight with GLUT4 antibodies (1:100) at 4 °C. After removing the primary antibodies, cells were washed with PBS 6 times for 5 min and incubated with secondary antibody Alexa Fluor® 488 (1: 1000) at room temperature for 1 h and visualized under a fluorescence microscope (BZ-X710).

2.8. Immunofluorescence image analysis

Fluorescence intensity measurements of the obtained fluorescence micrographs were performed using ImageJ software ver. 1.53i (National Institutes Health, Bethesda, MD); [16]. The captured image was separated into RGB channels, and the immunofluorescent staining intensity of GLUT4 was measured in the green channel. We measured the intensity of

green fluorescence (GLUT4) in whole cells, as well as inside the cells and nuclei, and calculated the intensity of green fluorescence in the plasma membranes from whole cells and within cells, as well as the intensity of green fluorescence in cell cytoplasm and nuclei. Finally, we measured the intensity of the plasma membrane to cytoplasm GLUT4 immunofluorescence ratio. Ten cells were analyzed per condition.

2.9. Statistical analyses

Data are presented as means ± standard errors of the means. The differences between vibration groups were analyzed using Dunnett's multiple comparison tests, where the non-vibration group that was not treated with insulin was considered the control group. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using JMP® 15 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Uptake of 2-DG by 3T3-L1 adipocytes is promoted by LFV and the most effective vibration intensity and duration is revealed

We assessed adipogenesis using oil red O staining (Figure 1A–F and a–f) and extracted the oil red O dye. There were no significant differences between groups (Figure 1G). Our investigation of the promotion of 2-DG uptake in adipocytes by LFV revealed that 1000 and 1500 mVpp groups significantly promoted 2-DG uptake ($p = 0.0372$ and 0.0018 , respectively) (Figure 1H). However, vibrations at 2000 mVpp did not promote 2-DG uptake. Accordingly, we concluded that the vibration intensity that induces 2-DG uptake in adipocytes most effectively lay between 1000–1500 mVpp.

Next, we attempted to determine the vibration duration that most effectively promotes glucose uptake. Vibration at 50 Hz and 1000 mVpp was applied for 40 min/d to 3T3-L1 adipocytes from day 8 to day 14 d past initial differentiation in all subsequent experiments. There was no difference in adipogenesis between groups (Figure 2A–E, a–e, and F).

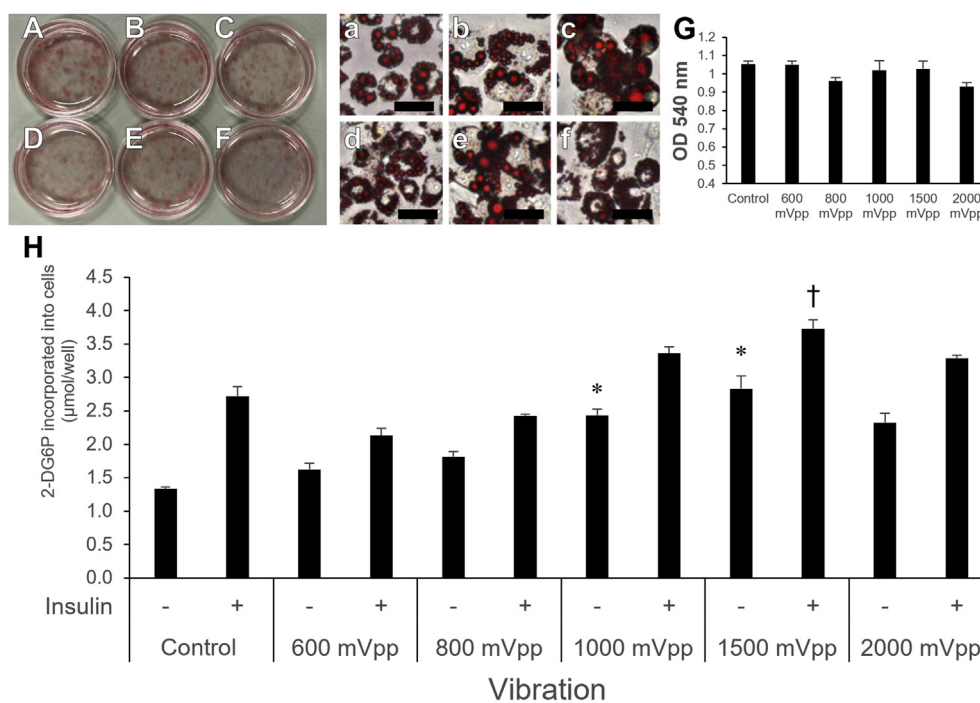


Figure 1. Oil red O staining in each group: Control (A, a), 600 mVpp (B, b), 800 mVpp (C, c), 1000 mVpp (D, d), 1500 mVpp (E, e), 2000 mVpp (F, f). Scale bar = 50 µm. (G) Dye extraction assay results of each group following oil red O staining. (H) 2-DG uptake in each group in the presence or absence of insulin. * $p < 0.05$ compared to the control without insulin treatment; † $p < 0.01$ compared to the control with insulin treatment; error bars represent standard error.

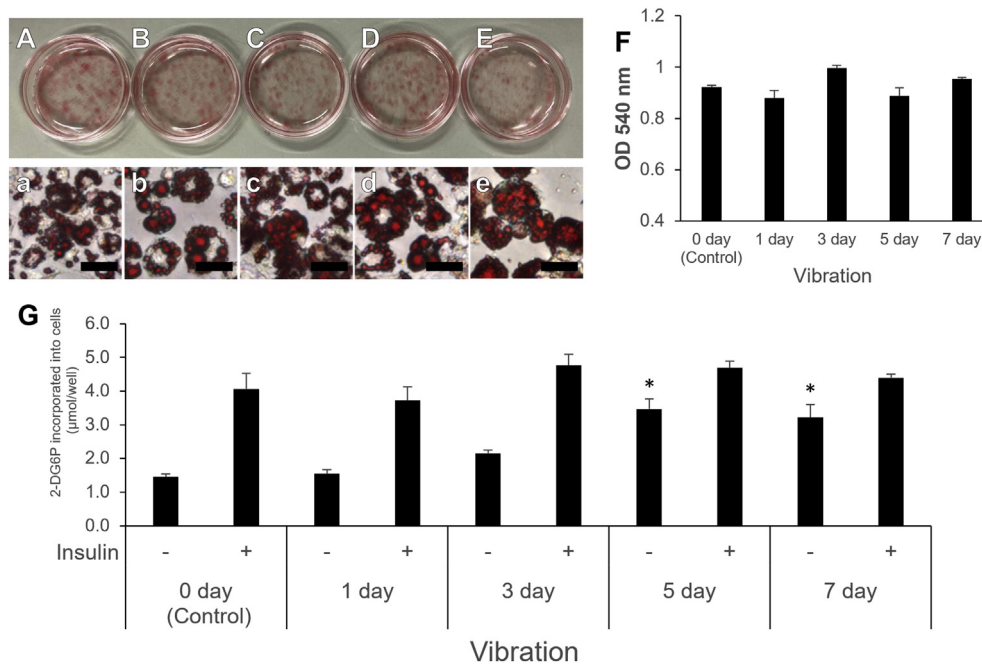


Figure 2. Oil red O staining for each duration: Control, 0-day group (A, a), 1-day group (B, b), 3-day group (C, c), 5-day group (D, d), 7-day group (E, e). Scale bar = 50 µm. (F) Dye extraction assay results following oil red O staining. (G) 2-DG uptake in each group in the presence or absence of insulin. * $p < .05$ compared to the control without insulin; error bars represent standard error.

Evidently, 2-DG uptakes of the 1-d and 3-d groups were similar to that of the control group, whereas those of the 5- and 7-d groups were higher than that of the control group ($p = 0.0169$ and 0.0452 , respectively). Thus, LFV at 50 Hz, 1000 mVpp, 40 min/d, over 5 d appeared to be

optimal for promoting glucose uptake in 3T3-L1 adipocytes. Furthermore, we evaluated the synergistic effect of LFV and insulin on glucose uptake in adipocytes, and we found that LFV and insulin did not exert a synergistic effect.

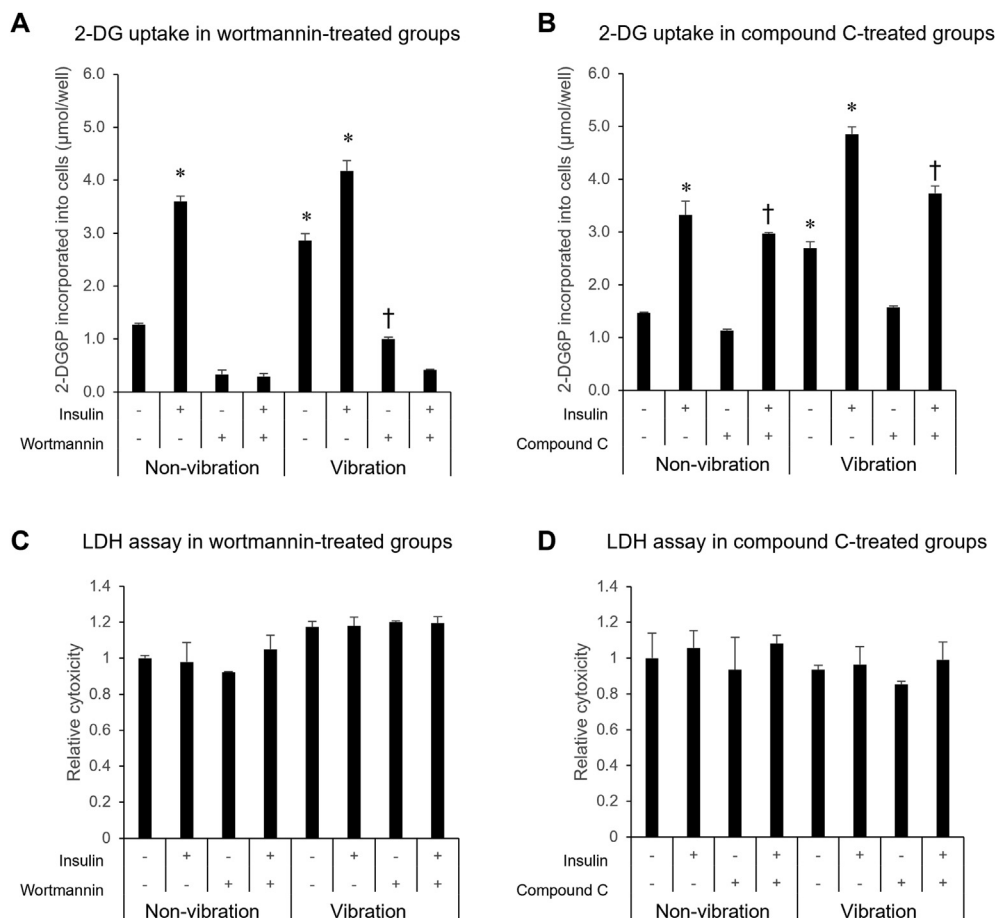


Figure 3. (A) 2-DG uptake in non-vibration and vibration groups treated with wortmannin. * $p < 0.01$ compared to the non-vibration without insulin; † $p < 0.01$ compared to the non-vibration with wortmannin. (B) 2-DG uptake in both non-vibration and vibration groups treated with compound C. * $p < 0.05$ compared to the non-vibration without insulin; † $p < 0.01$ compared to the non-vibration with compound C. Error bars represent standard error. (C–D) Relative LDH assay value in both non-vibration and vibration groups treated with wortmannin (C) or compound C (D), respectively. There were no significant differences between the non-vibration and vibration groups, regardless of either wortmannin or compound C treatment.

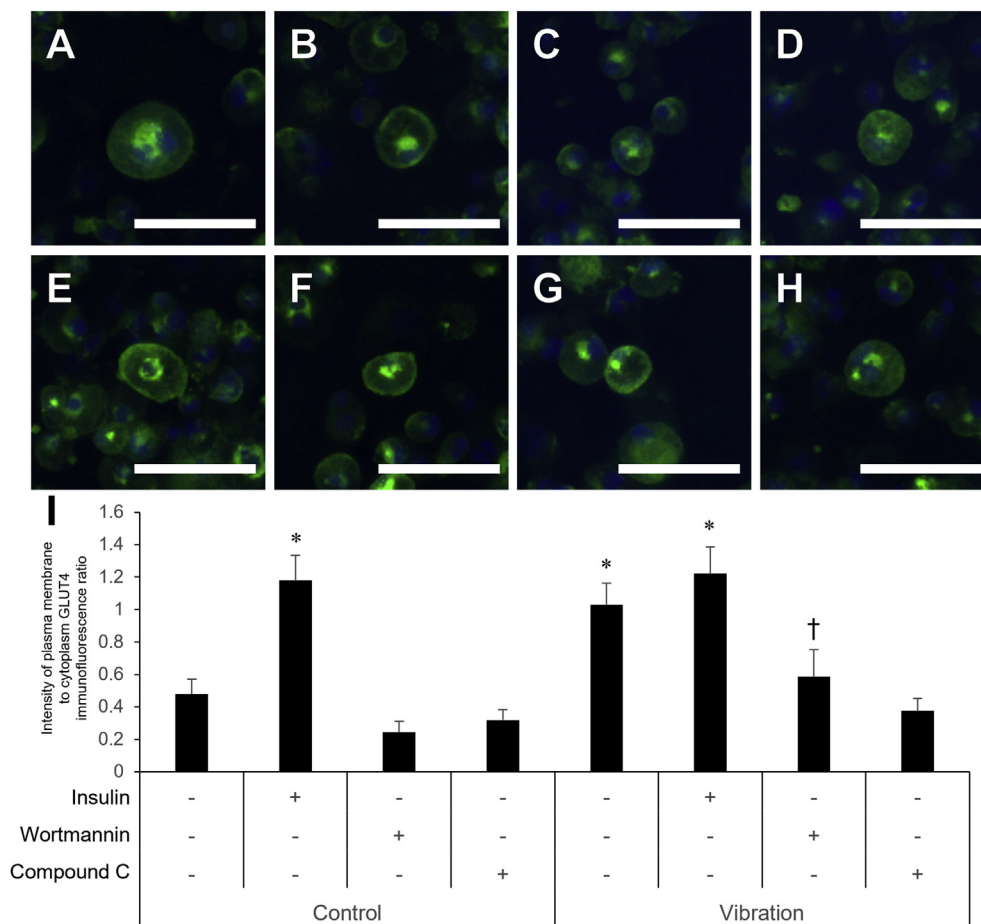


Figure 4. Immunofluorescence staining of GLUT4 under the following conditions: no-treatment (A); treated with insulin (B), wortmannin (C), and compound C (D) in the non-vibration group; vibration applied (E); and treated with insulin (F), wortmannin (G), and compound C (H) in the vibration group. (I) Intensity of the plasma membrane to cytoplasm GLUT4 immunofluorescence ratio. * $p < 0.01$ compared to the non-vibration; † $p < 0.01$ compared to the non-vibration with wortmannin; error bars represent standard error.

3.2. Wortmannin does not inhibit the 2-DG uptake; however, compound C inhibits the 2-DG uptake by LFV in 3T3-L1 adipocytes

We examined glucose uptake using inhibitors to elucidate the mechanism of glucose uptake by LFV. Wortmannin decreased 2-DG uptake in the non-vibration group regardless of insulin (Figure 3A). Furthermore, 2-DG uptake was decreased in the vibration group treated with both wortmannin and insulin, whereas 2-DG uptake was promoted in the vibration group treated with wortmannin alone compared to the non-vibration group treated with wortmannin ($p < 0.0001$). Additionally, no significant differences were observed between the LDH activities of the non-vibration and vibration groups, regardless of wortmannin.

The results of the experiment pertaining to the effect of compound C on 2-DG uptake showed that 2-DG uptake in the vibration group without insulin and compound C was higher than that in the non-vibration group ($p = 0.0248$; Figure 4B). Although compound C inhibited 2-DG uptake in the vibration group without insulin, there were no significant differences between 2-DG uptakes of the non-vibration and vibration groups. The results of the LDH assay indicated that there were no significant differences between the LDH activities of the non-vibration group and each vibration group, regardless of compound C (Figure 3C, D).

3.3. Immunofluorescence image analysis indicates that LFV promotes GLUT4 translocation

We investigated the promotion of GLUT4 translocation by LFV using immunofluorescence image analysis. Images indicating the intracellular localization of GLUT4 corresponding to each treatment in the vibration groups and the non-vibration are shown (Figure 4). Immunofluorescence staining of GLUT4 was observed in the whole cytoplasm of the non-vibration group (Figure 4A), whereas immunofluorescence of GLUT4

due to insulin was mainly observed at the plasma membrane (Figure 4B). When treated with wortmannin and compound C, GLUT4 was observed minimally at the plasma membrane and mostly in the cytoplasm (Figure 4C and D). GLUT4 was observed in the plasma membrane of the vibration group treated with insulin (Figure 4E and F). In the vibration group, GLUT4, which was observed in the plasma membrane when treated with wortmannin (Figure 4G), was mainly observed in the cytoplasm when treated with compound C (Figure 4H). Moreover, the intensity of plasma membrane to cytoplasm GLUT4 immunofluorescence ratio of the adipocytes was measured for each condition and compared to the non-vibration group (Figure 4I). The intensity of the GLUT4 immunofluorescence ratio in both groups treated with insulin was greater than that in the non-vibration group with no insulin treatment ($p < 0.0001$ and 0.0001 respectively), and the intensity of GLUT4 immunofluorescence ratio in the vibration group without insulin was greater than that in the non-vibration group ($p < 0.0001$). The intensity of GLUT4 immunofluorescence ratio in the vibration group treated with wortmannin was greater than that in the non-vibration group ($p < 0.0061$), but there was no difference between the vibration group treated with compound C and the non-vibration group.

4. Discussion

To the best of our knowledge, this is the first study to demonstrate the effect of LFV on AMPK-mediated glucose uptake in 3T3-L1 adipocytes. The results revealed that a LFV of 50 Hz at an intensity of 1000–1500 mVpp over 5 d was most effective at changing the adipocytes and promoting glucose uptake without changing the number of adipocytes or their maturation stage. Additionally, the results suggested that short, repeated exposures to LFV over several days were more effective than a single exposure.

In adipocytes, insulin-mediated GLUT4 translocation is accelerated by insulin secreted via the pancreas through the PI3-kinase pathway [17]. AMPK-mediated GLUT4 translocation is promoted by muscle contraction via a transient increase in intracellular calcium concentration or an increase in the AMP/ATP ratio [18]. Therefore, we investigated whether LFV increased 2-DG uptake via insulin-mediated or AMPK-mediated glucose uptake by observing intracellular location of GLUT4 using immunofluorescence. Wortmannin, an insulin-mediated glucose uptake (PI3K-Akt signal pathway) inhibitor [19], did not inhibit 2-DG uptake in the vibration group. However, compound C, a potent and highly selective inhibitor of AMPK activation [20], inhibited 2-DG uptake in the vibration group. Next, we investigated the intracellular location of GLUT4. A previous study has demonstrated that analysis of immunofluorescence images provides quantitative results which is equivalent to that of western blotting [21]. As indicated by immunofluorescence staining, the presence of GLUT4 was intense at the plasma membrane of the vibration group regardless of insulin treatment, indicating that LFV promoted GLUT4 translocation into the plasma membrane. Additionally, the presence of GLUT4 at the plasma membrane of the vibration group treated with compound C was minimal and there was no difference between the intensities of GLUT4 immunofluorescence ratios of the non-vibration and vibration groups treated with compound C. These results showed that compound C inhibited GLUT4 translocation in the vibration group, indicating that LFV promotes AMPK-mediated GLUT4 translocation.

Recently, it was reported that cells contain mechanosensors that perceive mechanical stimuli, such as vibration stimuli. Adipocytes convert mechanical stimuli, such as vibration stimuli, into physiological and pathological responses and gene expression via a process known as mechanotransduction [22]. This study indicated that LFV had the potential to activate mechanosensors, which promote AMPK-mediated GLUT4 translocation and glucose uptake. However, we were only able to demonstrate AMPK-mediated glucose uptake via an inhibition experiment using compound C and did not clarify the mechanisms and signal pathway associated with AMPK activation by LFV in adipocytes. Further studies are warranted to elucidate the signaling process related to AMPK activation by LFV in adipocytes.

Although we surmised that LFV and insulin together would be more effective in improving glucose uptake, the synergistic effect of LFV and insulin on glucose uptake was not elucidated in this study. DFU patients exhibit insulin resistance, whereby insulin-mediated glucose uptake does not work [2]. Thus, AMPK-mediated glucose uptake induced by LFV in adipocytes may potentially improve insulin resistance in DFU patients. Previous studies have reported that LFV at 600 mVpp for 10 min promotes local blood flow, through NO production and vasodilation [8, 9, 11]. However, this study found that a vibration intensity of 1000 mVpp applied over 5 d was required to promote glucose uptake. As the vibration conditions required for vasodilation and glucose uptake in adipocytes are different, this study suggested a new innovative approach that may correct hyperglycemia by increasing glucose uptake via the application of LFV at the wound site and also facilitate vasodilation by changing vibration conditions. This approach may promote the peripheral blood flow which is required to improve local insulin resistance and wound healing. Thus, further research is needed to investigate the relationship between glucose uptake and wound healing by LFV in animal diabetic models.

In conclusion, LFV at 50 Hz, 1000 mVpp, 40 min/d, over 5 d accelerated glucose uptake in 3T3-L1 adipocytes, and LFV promoted AMPK-mediated glucose uptake via an inhibition experiment using compound C. This study suggests that LFV has the potential to promote DFUs healing by promoting peripheral blood flow associated with improving hyperglycemia.

Declarations

Author contribution statement

Daijiro Haba, Gojiro Nakagami, Takeo Minematsu: Conceived and designed the experiments; Performed the experiments; Analyzed and

interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hiroimi Sanada: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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