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ABSTRACT

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Special Lecture

Intravital imaging dissecting immune cellular dynamics and identifying novel pathogenic cell types in vivo

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During the last decade, intravital optical microscopy has launched a new trend in the field of biology. By using this advanced imaging technique, we have established a new system for visualizing in situ behavior of a diversity of living cells within intact tissues and organs. Among them, we succeeded in visualizing the various dynamic phenomena within bones, where various kinds of immune cells are produced and function, although poorly analyzed by conventional methodology such as histological analyses with decalcified sections. We have so far identified the real modes of migration, differentiation, and function of bone-destroying osteoclasts, special kind of macrophages responsible for bone and joint erosions. In addition to the application to animal experimental models, we are currently trying to adapt this technique for evaluating disease status in local foci of human patients. In this presentation, I will present the recent update on intravital imaging studies on immune and other systems for clarifying in vivo behaviors of cell and tissue dynamics.

Symposium 1: Recent Advances in the Study of Microcirculation in Tumor and Immune Systems

Correlation of vascular cell adhesion molecule-1 expression in the colonic mucosa with mucosal inflammation and clinical course in ulcerative colitis

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Aim: Vascular cell adhesion molecule-1 (VCAM-1) is a 110 kDa transmembrane glycoprotein that is expressed in cytokine-activated endothelium and interacts with integrin VLA4 (α4β1) on the surface of leukocytes to promote leukocyte adhesion and signal transduction. Given the usefulness of α4 integrin inhibitors in the treatment of ulcerative colitis (UC), it is obvious that VCAM-1 is associated with the pathogenesis of UC. However, the association between the clinical course and mucosal VCAM-1 expression is still unclear in UC. In this study, we investigated the relationship between VCAM-1 expressions in the colonic mucosa and the clinical course of UC patients, and examined the role of VCAM-1 in the pathogenesis of UC.

Methods: Fifty-nine patients with UC in remission who were followed up for 2 years in Kyoto Prefectural University of Medicine were included in the study. All patients underwent biopsy from the rectum during colonoscopy, and mRNA expression of UC-related cytokines and VCAM-1 were quantified by real-time PCR. We examined the comparison between patients with subsequent relapse (relapse group) and those who remained in remission (remission group), and the correlation between VCAM-1 and endoscopic severity, histologic activity, or other adhesion molecules. This study was approved by the ethics committee of the Kyoto Prefectural University of Medicine (approval number: ERB-C-464-8).
Results: Twenty-five patients (42.4%) had relapses, and VCAM-1 expression was significantly increased in the relapse group ($p < 0.0001$). VCAM-1 expression increased in correlation with severity of endoscopic classification. VCAM-1 expression was significantly increased in histologically active mucosa compared to inactive mucosa ($p = 0.0235$) and correlated strongly with the expression of adhesion molecules such as MAdCAM-1, ICAM-1, ICAM-2, PCAM-1, and P-selectin ($r > 0.7$).
Conclusion: In the rectal mucosa of UC, VCAM-1 expression was highly correlated with mucosal inflammation and with other adhesion molecules, suggesting that VCAM-1 was associated with the clinical course and pathophysiology of UC.

Xenophagy and neovascularization in *Helicobacter suis*-infected gastric mucosal lymphoid follicle and MALT lymphoma

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As to the formation of the lymphocyte aggregation and angiogenesis leading to the gastric MALT lymphoma by Helicobacter species, little is known about the initial pathogenetic process following the infection. We have recently performed a study focused on the relation of the xenophagy to angiogenesis during *Helicobacter suis* (*H. suis*) infection.
Materials and Methods: Female C57/B mice were infected with *H. suis* for 3 months. Histological and Immunohistochemical study was performed using the antibody against *H. suis*, CD31, cleaved caspase 3, VEGF-A, VEGF-C, and 33D1 as a marker of dendritic cell and autophagy-regulating proteinase 4 (Atg4). In addition, ruthenium-red en bloc staining followed by Epon embedding was also done for electron microscopic observation and observed by JEOL 1200 EX- II electron microscopy at an accelerating voltage of 80 kv. This study was approved by the ethical committee of Kitasato Institute Hospital.
Results: In *H. suis*-infected mice, Atg-4 immunoreactivity was detected in the parietal cells near the lymph follicles. The autophagic vacuoles containing the bacteria were found within the cytoplasm of the parietal cell by electron microscopy. The debris of the bacteria were seen in the lamina propria mucosae near the parietal cells and the dendritic cells were also found in these areas surrounded by lymphocytes. VEGF-A immunoreactivity was recognized in these dendritic cells. Premature capillaries and lymphatic vessels were seen within the lymphocyte aggregation.
Conclusion: *H. suis* processed by xenophagy was shown to induce the dendritic cell activation, and angiogenesis related to the lymphocyte aggregation and the MALT lymphoma formation.

Symposium 2: Involvement of Lymphatic Microcirculatory Systems in Pathogenesis of Pathological Protein Aggregation and Metabolic Diseases

Water intake increases mesenteric lymph flow and mediates ATP release from myofibroblast cells

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Adequate water intake has long been recommended for maintaining health. It is well known that the lymph flow in small intestine is the greatest part of throughout the body. Therefore, we examined the effects of absorbed water in the jejunum on transported through mesenteric lymph vessels. Our previous study showed that water intake increased mesenteric lymph flow and the total flux of albumin, long-chain fatty acids, and innate lymphoid cells (ILC)3-dependent interleukin-22 (IL-22) in rats. Classic concepts suggest that water-soluble small molecules travel to the liver via the portal vein; however, our study showed the higher permeability of albumin-mediated transport in mesenteric lymph vessels of the jejunum, especially its upper part. IL-22 released from the ILC-3 is also transported through mesenteric lymph in collaboration with the albumin-mediated movement of consumed water. Subsequently, we investigated the effects of shear stress stimulation on jejunal physiological function and immunological regulation.
Shear stress stimulation of the cultured rat intestinal myofibroblast cells induced ATP release via an activation of cell surface F1/F0 ATP synthase. In addition, ATP produced podoplanin expression in the intestinal epithelial cells. Water intake accelerated immunohistochemical expressions of podoplanin and IL-22 in the interepithelial layers and lamina propria of the jejunum. ATP dose-dependently increased IL-22 mRNA expression in ILC-3, which are housed in the lamina propria.
In conclusion, water intake-mediated shear stress stimulated ATP release from myofibroblast cells, and then stimulation-dependent ATP considered that maintains higher tissue colloid osmotic pressure in the jejunal microcirculation through podoplanin upregulation in the interepithelial layers. In addition, ATP induces IL-22 mRNA expression in ILC-3 in jejunal villi, which may contribute to the regulation of mucosal immunity in small intestines. The conducted study was approved by the ethics committee of our institution.

Activation of innate immune system in experimental small intestinal ischemia–reperfusion injury

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Background and Aim: Toll-like receptors (TLRs) recognize the components of pathogenic microorganisms and trigger the activation of innate immunity. Myeloid differentiation primary-response 88 (MyD88), a common adaptor protein to transduce activation signals, induces the assembly of the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome, leading to the cleavage of pro-caspase-1 to the cleaved form of caspase-1, which promotes the processing of pro-interleukin-1β (IL-1β) into mature IL-1β. In this study, we aimed to investigate the activation mechanism of innate immune system in small intestinal ischemia–reperfusion (I/R) injury in mice.
Methods: Wild-type mice, MyD88 knockout mice, and cyclo-oxygenase-2 (COX-2) knockout mice were subjected to small intestinal I/R injury. I/R-induced small intestinal injury was characterized by infiltration of inflammatory cells, disruption of the mucosal epithelium, destruction of villi, and increases in myeloperoxidase activity and mRNA levels of inflammatory cytokines. All experimental procedures were approved by the Animal Care Committee of Osaka City University Graduate School of Medicine (approval number: 16027).
Results: MyD88 deficiency aggravated the severity of I/R injury, as assessed using the histological grading system, measuring luminal contents of hemoglobin, and counting apoptotic epithelial cells. I/R significantly enhanced COX-2 expression and increased PGE2 concentration in small intestine of wild-type mice, which were markedly inhibited in MyD88 knockout mice. COX-2 knockout mice were also highly susceptible to small intestinal I/R injury. Exogenous PGE2 reduced the severity of the injury in both MyD88 knockout mice and COX-2 knockout mice to the level of wild-type mice. I/R also significantly increased the mRNA levels of NLRP3 and IL-1β and increased the protein levels of both cleaved caspase-1 and mature IL-1β.
Conclusion: Activation of MyD88-dependent pathway and NLRP3 inflammasome may play an important role in experimental small intestinal I/R injury.

The correlation of choroidal pulsatility and vascular resistance with Cardio-Ankle Vascular Index

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Diabetic retinopathy (DR), a prevalent microvascular complication of diabetes, is linked with an increased risk of cardiovascular morbidity and mortality in type 2 diabetes mellitus (T2DM). Furthermore, arterial stiffness, which is a recognized predictor of cardiovascular risk, has been associated with DR in T2DM patients. The Cardio-Ankle Vascular Index (CAVI), measuring arterial elasticity from the aorta to the ankle, is influenced by coronary risk factors. Increases in these factors are reflected by elevated CAVI values, suggesting a higher probability of cardiovascular disease.
Laser Speckle Flowgraphy (LSFG) is a noninvasive technique for assessing ocular circulation, tracking the movement of erythrocytes within the eye, and providing data on vascular health through pulse waveforms and pulsatility. LSFG's capability to measure pulsatility, or beat strength (BS), allows for the analysis of blood flow dynamics that are modulated by cardiac cycles, irrespective of heart rate. The beat strength over the temporal average of the mean blur rate (BOM), which represents blood flow resistance, is an LSFG waveform parameter derived from frequency analysis. This is analogous to the pulsatility index utilized in Doppler ultrasound to assess blood flow resistivity in systemic vascular diseases.
These ocular circulation data obtained by LSFG may be crucial diagnostic tools that identify microvascular changes that indicate broader cardiovascular health issues, as evidenced by their relationship with conditions like retinal vein occlusion and macular degeneration. However, the association between ocular circulation and CAVI in patients with DR remains to be fully elucidated.
In this symposium, we would like to discuss the relationship between choroidal pulsatility in DR patients and systemic vascular conditions, with a special focus on CAVI.

Spatial heterogeneity of bone marrow endothelial cells unveils a distinct subtype in the epiphysis

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Bone marrow endothelial cells (BMECs) play a key role in bone formation and hematopoiesis. Although recent studies uncovered the cellular taxonomy of stromal compartments in the bone marrow (BM), the complexity of BMECs is not fully characterized. Previous research has focused on the metaphyseal and diaphyseal regions of the bone, while details about the endothelial cells in the epiphyseal region of the bone remained unclear. Here, using single-cell RNA sequencing, we defined a spatial heterogeneity of BMECs, and identified a new capillary subtype, termed type S (Secondary ossification) endothelial cells (ECs), exclusively existing in the epiphysis. Moreover, detailed histological observations showed that the type S vessels exhibit different morphologies from the vessels in metaphysis and diaphysis. We also showed differences in the localization and expression intensity of VEGF (vascular endothelial growth factor) receptors (VEGFR1-3), as well as differences in proliferation ability. Further evaluations on the epiphysis were conducted using mice with tamoxifen-inducible

endothelial-specific knockouts of VEGFR2 and DLL4 (Delta-like ligand 4). Mutant mice showed severely impaired angiogenesis and osteogenesis especially in the epiphysis. Type S ECs possessed unique phenotypic characteristics in terms of structure, plasticity, and gene expression profiles. In addition, genetic experiments showed that type S ECs atypically contributed to the acquisition of bone strength by secreting type I collagen, the most abundant bone matrix component. Moreover, these cells formed a distinct reservoir for hematopoietic stem cells. These findings provide the landscape for the cellular architecture in the BM vasculature, and underscore the importance of epiphyseal ECs during bone and hematopoietic development. In the future, our findings may lead to the discovery of new therapeutic options for bone diseases such as LCPD and osteoporosis. This research is approved by the institutional ethics committee.

Free Paper

Anti-oxidative Kampo formula can prevent development of nephropathy

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Hyperglycemia in diabetes increases reactive oxygen species production and resultant oxidative stress causes diabetic complications. Anti-oxidative treatment against diabetic complications by vitamin E, a representative anti-oxidative, is not yet established. Recently, it was reported that several Kampo (Chinese herbal) formulae possess strong anti-oxidative potency (Sato, Journal of Natural Medicines, 2018). Kampo formulae might be effective to diabetic complications without side effects.

The purpose of this study was to investigate whether a representative anti-oxidative Kampo formula, Tsudosan prevents diabetic nephropathy, one of major diabetic complications. Wistar rats were used after induction of diabetes by STZ injection (50 mg/kg). Rats were divided into seven groups including normal control. Each group was fed with standard diet mixed with powdered drug, whose dose was determined from body weight proportional to human dosage (diabetes without medication, diabetes with: Tsudosan, Tsudosan and SGLT2 inhibitor [forxiga], forxiga, Vitamin E, Saireito, and non-diabetic normal control). Glomerular filtration rate and 24-h protein excretion as indices for nephropathy were measured 25 weeks after beginning of drug administration and then rats were killed for renal tissue staining of 8-OHdG, oxidative stress, and DNA damage marker. Ratio of 8-OHdG stained area to a glomerular area was measured. The experimental protocols were approved by the Committee on Animal Research of Nozaki Tokushukai Hospital Institute and the care of all animals used in these experiments complied with the guidelines of the National Institute of Health.

There was no significant difference in glomerular filtration rates but Tsudosan and forxiga reduced protein excretion significantly compared with diabetic group without medication (34.0% and 66.7%, respectively, $p < 0.05$). Tsudosan and vitamin E significantly reduced area ratio of 8-OHdG (81.1% and 88.4%, respectively, $p < 0.05$). Taken together, these results indicate that a Kampo formula as an anti-oxidant, Tsudosan is more effective than vitamin E to prevent diabetic nephropathy.

Diffusion is a major mechanism involved in dextran transport through perivascular space

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Background: Perivascular flow in the brain, also known as glymphatic system, may serve as a clearance mechanism of large molecules, including amyloid beta (Aβ). We previously reported that Aβ perfused onto the cerebral cortex could be transported to the parenchyma around capillaries through perivascular space of perforating blood vessels (IJMS 2022). However, the detailed mechanisms under physiological conditions have not been completely elucidated. In this study, the dynamics of intracranially administered dextran was observed using a two-photon microscope.

Methods: A cranial window was placed in the brain of a C57BL/6 mouse (CLEA, Japan), and the procedure was performed under isoflurane anesthesia. Mice are continuously injected 40 kD, 110 kD dextran (100 μM each), or 2000 kD dextran (10 μM) were injected into the brain surface after head window creation, and continuous imaging was performed every minute from immediately after the start of injection until 30 min later. This study was approved by the Osaka City University Animal Ethics Committee.

Results: The movement of dextran from the superficial layer to the deep layer around the blood vessel over time could be observed for 30 min immediately after the measurement. There was no temporal delay in the dynamics of perivascular dextran between arteries and veins, and similar changes were observed in the brain parenchyma. Furthermore, as the molecular weight increased, migration was delayed. Diffusion rate is known to be inversely proportional to the square root of molecular weight, suggesting that diffusion rather than convection is the main mechanism for dextran movement along blood vessels.

Conclusion: Diffusion was largely involved in the movement of molecules in the perivascular space in both arteries and veins, and no significant convection was observed.

Assessment of the intestinal circulation for practical surgery

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Aim: In emergency operations for intestinal ischemia, it is often difficult for surgeons to decide the indication of resection and

the range of the resection. Fluorescence of indocyanine green (ICG) (wavelength 830 nm, near infrared) is usually used for assessment of the intestinal circulation in daily practical surgery. This study is aimed to compare visibility of the fluorescence of ICG and that of the fluorescein (wavelength 520 nm, visible) which was reported to suitable for microcirculatory assessment.
Methods: Eight 5-month-old, female pigs were administered isoflurane-maintained general anesthesia. Two segments of the small intestine and two intestinal anastomoses (with and without 2 cm ischemic part) were created in each pig. Six segments in eight pigs were clamped for 4 h and 10 segments in eight pigs were clamped for 7 h and they were de-clamped after administration of ICG (0.05 mg/kg) and the fluorescence was detected using HyperEye Medical System Plus (HEMS+). In four pigs, fluorescein (7 mg/kg) was administered and the fluorescence was detected by HEMS+. The pattern of fluorescence was assessed by Bulkley classification (Normal, Fine granular, Patchy, Perivascular, and Nonfluorescent). This study was approved by the Hungarian government office (approval number PE/EA/491-5/2020).
Results: Ischemia margins at the anastomoses were clearer in the fluorescein fluorescence than that of ICG. ICG fluorescence assessments of six segments after 4 h clamp were as follows: Five Fine granular and one Perivascular. In 10 segments after 7 h clamp were assessed as follows: Three Patchy, four Perivascular and three Nonfluorescent. All the intestinal segments after the 7 h clamp had partial or total area without reperfusion. All the three segments assessed as Perivascular by ICG fluorescence were assessed as Patchy by fluorescein fluorescence.
Conclusion: Difference of the fluorescence appearance between ICG and fluorescein may be explained by fluorescein ability of showing serosal superficial microcirculation.

Succinate augments inflammation in OVA-induced diarrheal mice models

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Introduction: Recently, succinic acid is said to play an important role in many kinds of inflammation in various organs through succinate receptors (SUNCR1/GPR91) that are expressed in quite a few varieties of cells both in rodents and humans. Our study focuses on how it works on OVA-induced diarrheal mice model.
Methods: We used 4–5 weeks aged female BALB/c mice for diarrheal model. We divided the diarrheal models into two groups, one is succinate-prescribed test group and the other is 1.8% NaCl-prescribed group as test control. Succinic disodium solution in Milli-Q water was given to each by gavage 1 h before the challenge test. All the diarrheal models received challenge tests with the same amount of OVA/PBS mixture and the number and features of feces were investigated. On the fifth day of challenge test, each intestinal organ was collected both for pathological study and RT-PCR. The blood sample, the feces from cecum and colon were collected, and we analyzed the quantity of organic acids and SCFAs in blood and stool using LCMS.
Results: IL-4, IL-13, and both intraepithelial mast cell-specific protease (Mcpt-1) and connective tissue mast cell-specific protease (Mcpt-4) were significantly elevated in stomach of OVA models with succinate by 12.7–102.7, 4.4–41.6, 2.8–112.5, and 1.4–1.8 folds each. IL-4, IL-13, Mcpt-1, and Mcpt-4 were far more elevated in cecum of diarrheal model with succinate by 17.6–74.0, 77.9–484.3, 111.1–373.9, and 105.3–419.6 folds each. As for SUNCR1 expression, only diarrheal model with 1.8% NaCl showed significant elevation in stomach by 2.9–7.9 folds and slightly in cecum by 0.5–2.7 folds. Serum butyrate was significantly lower in OVA diarrheal models with succinate compared to test control.
Conclusion: Succinate possibly aggravates allergic reaction via Mcpt-1 and Mcpt-4 expression in stomach and cecum with lowering butyrate concentration in serum and cecum feces.

Applicants' Presentation for Young Investigator Award

Effects of succinic acid on the gut immunity of mice

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Introduction: Metabolites in the human body interact in a variety of ways to influence homeostasis and many diseases. Alterations in the composition and metabolism of the gut microbiota have been established as a contributing factor in inflammatory bowel disease (IBD), but the mechanisms remain unclear. It has been reported that succinate accumulates in the bodies of IBD patients and that elevated concentrations of succinate promote inflammation via the succinate receptor 1 (SUCNR1). However, the mechanism of how succinate is involved in the pathogenesis of IBD remains unclear; IBD is known to increase lymphocyte adhesion to vascular endothelium via adhesion molecules in the intestinal tract. We hypothesized that succinic acid might modulate lymphocyte adhesion by enhancing adhesion molecules. We aimed to investigate the effects of succinic acid on intestinal microcirculation.
Methods: Male wild-type mice (C57BL/6) were used in the study. Succinic acid was administered intraperitoneally, and 24 h after administration, small intestinal terminals were harvested and evaluated histologically. Succinic acid was filled into the intestinal tract and fluorescent dye (CFSE) was slowly injected through the jugular vein. Microcirculation of the small intestine was observed by confocal laser scanning microscopy (CLSM). This study was conducted with the approval of the Ethics Committee of the researcher's institution.
Results: Succinic acid-treated mice showed shortening of villi length and lengthening of the crypts in the ileum, and increased expression of VCAM-1 in mRNA, compared with the subject mice. Succinic acid-treated mice showed leukocyte adhesion in

the intestinal mucosa about 30 min after administration compared with control mice.
Conclusion: Succinic acid may be involved in intestinal inflammation in IBD by promoting leukocyte adhesion. Further studies are needed to elucidate the exact mechanism.

Spatiotemporal structure of spontaneous fluctuations in brain capillary diameter in the anesthetized mouse cortex

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Background: Capillaries in the brain change their diameter in response to neural activity. However, measuring these subtle changes can be difficult. In this study, we examined spatiotemporal variations in capillary diameter and the surrounding cells using two-photon imaging datasets.
Methods: We used previously published data from fluorescent images of capillary diameters labeled through blood plasma and vascular mural cells in the anesthetized mouse cortex ($N = 6$). A total of 122 capillaries with 5500 measurement points were extracted and MATLAB was used to determine their diameters and the morphological characteristics of the mural cells along the capillaries.
Results: We found that the spatial variations in resting capillary diameters were correlated with the morphological variations in the mural cells located outside of the capillary. These variations in diameters showed a spatial frequency of 15–20 per micrometer along the capillaries, which also matched the morphological variations in the mural cells (i.e., cell body and processes). However, the temporal fluctuations in resting capillary diameters showed much higher spatial frequencies (i.e., 3–10 per micrometer) and were independent of the coverage of mural cells. We also found that the diameter fluctuations did not propagate along the capillaries.
Conclusion: In conclusion, the present study revealed distinct patterns of capillary diameter variations that correlated with the morphological diversity of the vascular mural cells.

Lower extremity exercise during prolonged sitting prevents impaired oxygen extraction in the gastrocnemius muscle: A randomized crossover trial

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Aim: Prolonged sitting is known to induce metabolic and vascular disorders. However, the effects on oxygen delivery between capillaries and muscles (muscle oxygen extraction) are unknown. The purpose of this study was to clarify the effects of prolonged sitting on oxygen extraction in the gastrocnemius muscle.
Methods: The participants were 12 healthy young men. The study design was a randomized crossover design with two experiments. Protocol 1 consisted of a comparison of a 3-h supine (CON) and seated (SIT) condition. Protocol 2 consisted of a 3-h sitting (SIT) and a lower limb movement condition (ACT) during sitting. The near-infrared spectroscopy device was placed over the skin of the medial head of the gastrocnemius muscle, and a pneumatic cuff was placed on the distal end of the corresponding thigh; once every hour, a baseline assessment of tissue oxygen saturation (StO_2) was recorded, after which the pneumatic cuff was inflated for 5 min. The area under the curve was calculated from the response to a decrease in StO_2 during the occlusion phase, which was used as an index of muscle oxygen extraction (StO_2 AUC). The obtained indices were compared by repeated two-way analysis of variance (time × group factor). This study was conducted with the approval of the Ethics Committee of Niigata University of Health and Welfare (approval number: 19004–230310).
Results: There was significant interaction between StO_2 AUC under CON and SIT conditions, with significantly lower values under SIT (CON; 78 925 ± 1342 vs. SIT; 42 090 ± 1139 a.u., $p < 0.01$); there was also a significant interaction between under SIT and ACT conditions, with significantly higher values under ACT (SIT; 49 773 ± 1406 vs. ACT; 62 214 ± 1860 a.u., $p < 0.01$).
Conclusion: Impaired oxygen extraction in the gastrocnemius induced by prolonged sitting can be inhibited by lower extremity exercise.