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博士（スポーツ科学）

ホホバオイルとウインターグリーンオイルの事前塗布は、運動誘発性炎症を緩和する

Pre-Application of Jojoba and Wintergreen Oils Relieves Exercise-Induced Inflammation

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Chapter 1.

Introduction

1.1. Sports Injury

Even if the exercise intensity is at an amateur level, there is a high risk of pain in various parts of the body due to fatigue or excessive movement due to training (1). Additionally, athletes performing competitive sports exert their strength at peak levels, aiming for podiums and medals, and exerting endurance activities at levels close to their own limits. Therefore, sports injuries are an occupational risk for elite athletes (2). Sports injury is roughly divided into traumatic and overuse injuries. Traumatic injury is caused by a large external force, and a typical example is a sudden injury such as dislocation, bone fracture, sprain, ligament injury, or sprained finger. In contrast, overuse injury is caused by overuse and persistent load and includes tennis elbow (lateral epicondylitis), baseball elbow (medial epicondylitis), stress fractures, and Achilles tendonitis.

Exhaustive or unaccustomed exercise also causes acute muscle damage, which causes delayed-onset muscle soreness (DOMS) (3). DOMS has been reported to cause muscle weakness, muscle swelling, and a reduced range of motion (4, 5). The mechanism underlying this condition might be mechanical damage to muscle fibers and subsequent inflammation; that is, after intense exercise, elevated plasma myoglobin, creatine kinase (CK), inflammatory cytokines (interleukin [IL]-12p40, IL-6), a chemokine (monocyte chemoattractant protein [MCP]-1), and myeloperoxidase (MPO) indicate neutrophil

activation (6-9). MPO is an enzyme that is rich in neutrophils and converts hydrogen peroxide to the more toxic hypochlorous acid and causes oxidative stress.

1.2. Inflammation and Oxidative Stress Caused by Exercise

High intensity or prolonged muscle contraction activity damages skeletal muscles. There are also many commonalities between the symptoms of exercise-induced muscle damage and the classic signs of local inflammation (pain, fever, redness, swelling, and loss of function). Inflammation is an important process in muscle repair and regeneration (10, 11). However, excessive exercise induces inflammation, and muscle cells also release various cytokines that cause inflammation (12). Chemokines induce the invasion of phagocytic cells into damaged skeletal muscle after exercise. Inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-8, which are produced during inflammation, cause the migration and invasion of phagocytic cells to produce reactive oxygen species (ROS) via NADPH oxidase and MPO. Inflammatory cytokines also directly enhance ROS production in the mitochondria.

Exercise causes an increase in pro-inflammatory cytokines, in turn increasing muscle oxidative stress, which can lead to a vicious cycle and further increase in inflammation (10). In contrast, ROS are involved in the secretion of inflammatory cytokines. In this way, inflammation and oxidative stress are closely related, and oxidative stress that exceeds the controllability of antioxidants, such as antioxidant enzymes and vitamins, causes injury to the body and diseases. For example, oxidative stress is involved in various pathophysiological conditions such as cardiovascular disease, cancer, Alzheimer's disease, diabetes mellitus, ischemia/reperfusion injury, rheumatoid arthritis, and aging (13-16).

In contrast, free radical production also occurs during normal cellular activity and is part of the natural physiological processes of all living organisms (13, 17). Free radicals usually act as both beneficial and toxic compounds (13). Many important physiological functions, such as muscle contraction and drug detoxification, require low levels of free radicals from ROS or oxidative stress. However, a dramatic increase in ROS during exhaustive exercise can damage cell membranes and adversely affect skeletal muscle performance, causing macromolecular damage and impaired cell function (17).

1.3. Sports Massage

Massage has been practiced since B.C. years (18, 19) and is a manipulative therapy developed in Europe. Currently, sports massage is performed for the purpose of fatigue recovery, conditioning, warm-up, performance improvement, treatment, and the prevention of sports disorders. It is believed that massage can be used to passively move and stimulate muscles to promote the circulation of blood and lymph, encourage metabolism in muscles, and eliminate muscle fatigue. Lubricants are used for massage. For example, those used as massage include oil, massage cream, and powder. When massaging for the purpose of pain relief or inflammation suppression associated with an overuse injury, a medicated cream containing a drug with analgesic and anti-inflammatory action, namely nonsteroidal anti-inflammatory drugs (NSAIDs), can be used. Topical anti-inflammatory therapy is an effective therapeutic strategy because it enables local analgesic and anti-inflammatory effects while minimizing systemic side effects (20). In Europe and America, sports massage is mainly carried out using various massage oils. The use of massage oil stimulates blood and lymph circulation. The use of

oil makes it possible to apply pressure to the treated area while gliding the hand. This is an effective movement against peeling off the adhered fascia and contributes to the effectiveness of sports massage. It is difficult in dry massage to apply the same technique to a wide range of areas. If essential oils are used together, the effect of aromatherapy can be expected in addition to the effect of massage.

Sports massage is widely used by athletes as part of their conditioning regimen. In fact, previous meta-analyses and systematic reviews have shown that massage can mitigate and prevent DOMS (21). Other studies have shown that massage as a warm-up improves muscle flexibility (21, 22); however, it does not seem to exert a positive effect on concentric calf peak force and performance in the sprint, drop jump, and vertical jump (22, 23). In addition, post-exercise massage has been reported to improve the exercise performance and recovery rate of bodybuilders after intensive exercise (4) and to relieve exercise-induced inflammation (24) and muscle pain (25). Thus, the benefits of using massage for conditioning are becoming apparent. In some countries that participate in the Olympics, not only medical doctors and physiotherapists but also massage therapists participate as members of the medical delegation team that is accompanied by the athlete (26). The International Olympic Committee website shows that elite athletes are actually incorporating sports massage into their conditioning. Callaghan also reported that at least 787 massages were performed at the Olympics in Barcelona in 1992 by physiotherapists and masseurs of the UK team (27). From these facts, it can be seen that from the 1990s to the present, sports massage has been considered useful for conditioning and has been utilized by athletes.

1.4. Sports Massage Using Essential Oil

In this study, sports aromatherapy massage was defined as a method of performing sports massage using essential oil diluted with a carrier oil. This method is based on Swedish massage (also known as classic massage), which is widely used worldwide and is performed using techniques such as effleurage, petrissage, friction, tapotement (rhythmic tapping), compression, and vibration. The reason for diluting the essential oils with carrier oil is to reduce irritation to the skin and to spread it evenly on the skin so that the essential oil is absorbed over time. Usually, aromatherapy massage to promote relaxation uses essential oil diluted with a carrier oil to approximately 1%. In contrast, in sports aromatherapy massage, essential oils diluted to a high concentration of 5–10% are used to achieve the expected action of essential oil components. Another method is to use essential oil in an undiluted solution. This method includes warming the skin with the heat of a drier and dropping a few drops of essential oil onto the affected area to allow it to penetrate. In this case, the purpose is to apply undiluted solutions such as wintergreen oil or clove oil to the skin to suppress back pain and menstrual pain.

As a scientific basis for applying essential oils to sports massage, for example, it is known that some essential oils have anti-inflammatory and antioxidative effects (28-33). When performing massage for the purpose of alleviating pain associated with overuse injury and suppressing inflammation, a medicated cream containing a drug with analgesic and anti-inflammatory action, such as indomethacin or diclofenac sodium might be used. Similarly, sports aromatherapy massage can be expected to reduce the inflammation caused by exercise by adding essential oil to the oil used in sports massage. Another way to apply essential oils to sports massage is to spread the essential oil in the room, but it seems that the effect is strengthened by using massage in addition to

inhalation. For example, a study investigating the anxiety-relieving effect of essential oils in cancer patients in the palliative care unit showed that the inhalation of aroma oil alone was not effective (34), but the effect of using massage in combination was significantly effective (35, 36). Aromatherapy massage is more beneficial for anxiety relief than massage using carrier oil without the use of essential oils (37). From these facts, it is considered that the physiological effects of essential oils are exerted by the transdermal absorption of active components.

Essential oils are natural volatile products extracted from various parts of the plant and contain natural compounds that are highly concentrated even in very small amounts with various aromatic components. The volatile constituents of essential oils are useful for the survival of plants and for odor-mediated communication between plants, but the synthesis of volatiles has a high metabolic cost. For example, volatile compounds that are released to the outside of the body through feeding damage are used as indicators to attract predators of herbivorous insects and parasitic plants (38-41). Insect-damaged plants synthesize volatile chemicals at the damaged site, which act as a poison to the prey to prevent further damage from spreading (42). It has also been reported that adjacent plants receive this volatile compound and induce the expression of defense response genes to increase resistance (43, 44).

1.5. Adverse Effects of Essential Oils Used in Sports Aromatherapy Massage

The essential oils used in sports aromatherapy massages have a low risk of adverse effects if some precautions are followed, but there are a few things to keep in mind when using them safely. In some people, a hypersensitivity reaction to essential oils can occur. If one is allergic or has sensitive skin, it is safer to perform a patch test in advance of

using as essential oil for the first time. In addition, before applying it to the skin, the essential oil needs to be diluted with a carrier oil. In addition, sun exposure after applying citrus essential oils such as bergamot, bitter orange, and lemon can cause spots. It is important to note this in competitions held outdoors during the daytime. In addition, essential oils should not be consumed (33). As a serious side effect, death in children who swallowed approximately 5 mL of wintergreen oil at one time has also been reported (45, 46). Finally, the current situation in Japan is that there are no regulations for essential oils. Here, the majority of essential oils are legally not included in medicines and cosmetics and are treated as miscellaneous goods. Further, in Japan, only a few essential oils (orange oil, mentha oil, cinnamon oil, clove oil, fennel oil, etc.) are listed in The Japanese Pharmacopoeia 17th edition (47); this means that only these essential oils are statutorily regarded as having medicinal properties. Drugs covered by insurance are subject to the responsibility of the pharmaceutical company if side effects occur, but there are no laws regulating the safety and effectiveness of the majority of essential oils. If any side effects occur from the use of essential oils, the user is responsible for their own care.

The essential oils that are categorized into miscellaneous goods do not have strict quality regulations, and thus, quality levels are considered diverse. Therefore, when purchasing essential oils, it is recommended to choose an essential oil that presents the production area, extraction site, extraction method, plant cultivation method, and specification of characteristic components based on gas chromatography-mass spectrometry (GC-MS) analysis. If possible, it is important to select high-quality essential oils that meet the Essential Oils Botanically and Biochemically Defined (E.O.B.B.D.®) standards. As such, E.O.B.B.D.® is the established quality standard for essential oils for medical aromatherapy.

1.6. Essential Oils and Doping

Doping is an action to enhance competitiveness by using substances and methods that are prohibited by the World Anti-Doping Agency (WADA) to purposely gain an advantage and achieve victory. The definition of doping is not limited to the intentional use of prohibited substances. It is prohibited to use various methods to enhance one's ability to compete, whether intentional or not, and the concealment of such violations is also defined as doping (48).

The WADA is an international surveillance agency established in November 1999 and publishes a revised annual list of banned substances called the World Anti-Doping Code (48) (Table 1). No essential oils or ingredients that violate these doping regulations are available in commonly sold essential oils. The wintergreen oil used in this study is also not a substance prohibited by WADA. Methyl salicylate is a widely used compound and is the main ingredient of compresses prescribed for pain.

Table 1. The 2020 list of prohibited substances of the world anti-doping code (48).

Prohibited substances
S0. Non-approved substances
S1. Anabolic agents
1. Anabolic androgenic steroids
2. Other anabolic agents
S2. Peptide hormones growth factors, related substances, and mimetics
S3. Beta-2 agonists
S4. Hormone and metabolic modulators
S5. Diuretics and masking agents
Prohibited medhods
M1. Manipulation of blood and blood components
M2. Chemical and physical manipulation
M3. Gene and cell doping
Substances and methods prohibited in-competition
Prohibited substances
S6. Stimulants
S7. Narcotics
S8. Cannabinoids
S9. Glucocorticoids
Substances prohibited in particular sports
P1. Beta-blockers

1.7. The Purpose of This Study

Sports aromatherapy massage might be more effective than massage alone, by adding the effect of essential oils to sports massage, and is considered useful for athlete conditioning. At present, there is insufficient scientific evidence regarding the use of essential oils in the sports field, and previous studies on aromatherapy have not fully verified the physiological effects of carrier oils. The reason for the lack of sufficient evidence in this area is that studies on humans have large individual differences; it is difficult to apply a certain amount of pressure because massage is a manual therapy, and consistent results are not easy to obtain. Therefore, in this study, animal experiments were conducted, and jojoba oil and wintergreen oil, which have been reported to have anti-inflammatory effects, were applied to the backs of mice for percutaneously absorption, and the following was examined.

- Verify whether jojoba oil or wintergreen essential oil applied before exercise affects endurance capacity during treadmill running
- Confirm the effect of jojoba oil on blood biochemical parameters after percutaneous absorption
- Verify whether jojoba oil or wintergreen oil applied before exercise can alleviate exercise-induced inflammation
- Evaluate the safety of the transdermal absorption of wintergreen oil based on blood biochemical data

Jojoba oil is a commonly used carrier oil among massage therapists due to its high safety. In addition, jojoba oil is less prone to oxidation than other oils, which makes it easier for individual athletes to use for sports massage. For these reasons, the biological action of jojoba oil was examined in this study and was selected as the carrier oil for

diluting wintergreen oil.

Chapter 2.

Acute Effects of Transdermal Administration of Jojoba Oil on Lipid Metabolism in Mice

2.1 Introduction

Plant-based therapies for many diseases have been studied for a long time. Research is currently being conducted to assess the application of plant-derived components such as extracts and essential oils for medical treatments (49, 50) and insecticides (51). Most importantly, aromatherapy and massage are used in clinical settings (52, 53) and in sports medicine (54) as complementary and alternative therapies. Aromatherapy massages are administered by diluting essential oils with carrier oils. For example, plant-derived oils (e.g. almond oil and jojoba oil) have been widely used as skin emollients and moisturizers (55-58).

Although numerous studies on massage therapy as a complementary or alternative therapy or in the context of sports medicine have been carried out, limited information is available regarding the benefits of massage oils or carrier oils for the skin. Furthermore, in several review articles, the type of massage oil used was not indicated (27, 54, 59-63). Hence, especially regarding aromatherapy research, the pharmaceutically active ingredients contained in each essential oil are expected to exert effects, whereas the effect of the carrier oil itself, used to dilute the essential oil, has been thought to be negligible. Similarly, studies on sports medicine primarily focus on the direct effect of the massage, because of the physiological effects of the massage oil itself

are often not adequately considered. The common carrier oils that are now used for aromatherapy massages are jojoba oil, grape seed oil, macadamia nut oil, and sweet almond oil. Among these, jojoba oil is one of the most widely used carrier oils worldwide.

Although jojoba oil is often categorized as an "oil" because of its pale yellow, transparent, liquid appearance, it is actually a liquid wax ester. Wax esters are generally straight-chain esters of mono-unsaturated long-chain fatty acids and fatty alcohols. The wax esters produced by jojoba are very similar to sebum produced naturally by the human skin. Human sebum is comprised of approximately 33% triglycerides, 28% free fatty acids, 25% wax esters, 10% squalene, 2% cholesterol esters, and 4% cholesterol (64). Further, wax esters exert moisturizing effects and impart softness to the skin. Jojoba (*Simmondsia chinensis*) is the only plant species known for synthesizing liquid wax, which constitutes approximately 40–60% of the dry weight of mature jojoba seeds (65). In general, plant-derived oils are rich in triglycerides; therefore, jojoba oil differs from other seed oils due to the fact that it contains primarily liquid wax, rather than triglycerides (66).

As mentioned above, jojoba oil is a wax ester, the same as human sebum components, and has been confirmed to be safe. Therefore, jojoba oil is one of the most commonly used carrier oil among massage therapists. In addition, jojoba oil is not oxidized easily and lasts longer than other carrier oils, which makes it easier for individual athletes to use for sports massage. Thus, jojoba oil is widely used as a carrier oil, but only limited information is available on its effects on blood biochemical parameters. This study aimed to investigate the effect of transdermal administration of jojoba oil on blood biochemical parameters in mice. As a result, the plasma non-esterified fatty acids (NEFA) levels were significantly increased by about 20% at 30 min after

topical application of jojoba oil. Therefore, to investigate the mechanism of increased plasma NEFA level after topical application of jojoba oil, this study further analyzed the expression levels of lipid metabolism-related genes in various tissues/organs.

2.2. Materials and Methods

2.2.1. Ethics Statement

All animals were cared for in accordance with Law No. 105 and Notification No. 6 of the Japanese Government, and all animal experiments were carried out with the approval of the Animal Experimentation Committee of Tokai University (permission #171096 and #181020).

2.2.2. Animals

Seven-week-old male hairless mice (Hos-HR-1) were purchased from Hoshino Laboratory Animals, Inc. (Bando, Japan) through Japan SLC, Inc. (Hamamatsu, Japan), and housed at the Department of Laboratory Animal Science, the Support Center for Medical Research and Education, Tokai University, for at least 1 week. All mice were 8 weeks of age (26.9–32.3 g; average = 29.8 g) during the experiments. All mice were housed under specific pathogen-free conditions with a standard commercial diet (Clea, Tokyo, Japan) and water was provided *ad libitum* until just before euthanasia. The mice were housed at 22–24°C with 50–60% relative humidity, under a light-dark cycle (lights on at 08:00 and off at 20:00).

2.2.3. Chemicals

Jojoba oil was obtained from Kenso-Igakusha (Yamanashi, Japan; Lot. GL15A).

Jojoba oil is a fatty acid-containing wax ester and is different from common vegetable oils, rich in triglycerides. Based on the manufacturer's package insert, the ingredients of jojoba oil used in this study are listed in Table 2.

Table 2. Fatty acid composition of jojoba oil.

Name	MW [g/mol]	Content [%]
Eicosenoic acid	310.51	73.4
Erucic acid	338.57	14.7
Oleic acid	282.47	08.3

MW, molecular weight.

2.2.4. Experimental Protocol

On the day of the experiment, all mice were randomly divided into two groups of six animals each, based on the different topical applications as follows: the naïve control group (26.9–32.3 g; average = 29.7 g) and the undiluted jojoba oil group (28.9–31.7 g; average = 29.9 g). Mice were topically administered 4 μL of jojoba oil per gram of body weight to the dorsal area, 30 min before euthanasia. The jojoba oil was evenly spread on the dorsal skin. After topical application, the mice were housed separately and monitored for adverse effects caused by oil application, in the form of skin rashes (erythema, dermatitis, etc.). Blood samples were taken using heparin from the heart under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan), and then isolated issues/organs were immediately frozen in liquid nitrogen. Plasma was obtained from blood samples by centrifugation at 1500 × *g* for 15 min at 4°C. These samples were stored at −80°C until analyses.

The sampling time was determined based on previous studies, wherein the time-dependent concentration of the active ingredient in blood was examined using hairless

mice (67) and humans (68), after transdermal absorption of the essential oil. On preliminary analysis, an increase in sampling time affected lipid metabolism. Hence, all tissues were sampled from 9 a.m. to 11 a.m., so as not to affect lipid metabolism in mice. Hairless mice were used because there was no risk of scratching the dorsal skin prior to transdermal administration, due to the process of hair removal.

2.2.5. Biochemical Analysis of Plasma

Plasma levels of albumin (ALB), blood urea nitrogen (BUN), creatinine (CRE), uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), total cholesterol (T-CHO), triglyceride (TG), phospholipid (PL), NEFA, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total bile acid (TBA), glucose (GLU), lactic acid (LA), and total ketone body (T-KB) were measured by Oriental Yeast Co., Ltd. (Tokyo, Japan).

2.2.6. RNA Isolation and Gene Expression Analysis Using Real-Time Polymerase Chain Reaction (Real-Time PCR)

Immediately after harvesting tissue/organ samples, each sample was frozen in liquid nitrogen and stored at −80°C until RNA extraction, including the liver, white adipose tissue (WAT) of the epididymis, skin, brown adipose tissue (BAT), plantaris muscle, and the heart. Based on the manufacturer's instructions, total RNA was extracted from the liver using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), total RNA from the heart, plantaris muscle, and skin were extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen), and total RNA from WAT and BAT were extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen).

The purity and the quality of total RNA from each sample were assessed based on the optical density 260:280 nm ratio, using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA). Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA), in accordance with the manufacturer's instructions. PCR was carried out using the StepOnePlusTM system (Applied Biosystems), using the Fast SYBR® Green Master Mix (Applied Biosystems). The cycling conditions were as follows: 10 s at 95°C for denaturation, followed by 45 cycles at 95°C for 5 s, 57°C for 10 s (annealing), and lastly, 72°C for 10 s. Data were normalized to β-actin levels as an internal standard, using the calibration curve method. The genes and primers used are listed in Table 3.

Table 3. Primers used for real-time polymerase chain reaction analysis.

Gene	Accession no.	Forward	Reverse
Atgl	NM_025802.3	TGTGGCCTCATTCCTCCTAC	TCGTGGATGTTGGTGGAGCT
Hsl	NM_010719.5	GCTGGGCTGTCAAGCACTGT	GTAACTGGGTAGGCTGCCAT
Lpl	NM_008509.2	CCAATGGAGGCACTTTCCA	TGGTCCACGTCTCCGAGTC
Cpt-1a	XM_006531658.3	CCAGGCTACAGTGGGACATT	AAGGAATGCAGGTCCACATC
Cd36	NM_001159558.1	CCGGGCCAACGTAGAAAACA	CCTCCAAACACAGCCAGGAC
FABPpm	NM_010325.2	AGCGGCTGACCAAGGAGTT	GACCCCTGCCACGGAGAT
FATP-1	NM_011977.4	GGCTCCTGGAGCAGGAACA	ACGGAAGTCCCAGAAACCAA
FATP-2	NM_011978.2	TTCGGGAACCACAGGTCTTC	GCAAGGCTTGTCCCATACCTT
FATP-3	NM_001316688.1	CAGCTCTACAGCCATGTTTCTGA	CAAAGATTCCTGGAGCCTGAGA
FATP-4	NM_011989.5	GGCTTCCCTGGTGTACTATGGAT	ACGATGTTTCCTGCTGAGTGGTA
FATP-5	NM_009512.2	TTTCTGGGGTTGGCCAAGTT	TGGCCAAGGTAGAAGCAGTG
FATP-6	NM_001081072.1	GGCTTGAGGATGCCGCTTA	GTACTCTGGGCTCATGCTATGAAGT
Fas	NM_007988.3	CCTGGATAGCATTCCGAACCT	AGCACATCTCGAAGGCTACACA
Acc-1	XM_011248667.1	ATTGGGCACCCCAGAGCTA	CCCGCTCCTTCAACTTGCT
Acc-2	XM_006530113.3	GGGCTCCCTGGATGACAAC	TTCCGGGAGGAGTTCTGGA
Scd-1	NM_009127.4	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Ppar-a	XM_006520624.3	TCTGTGGGCTCACTGTTCT	AGGGCTCATCCTGTCTTTG
Srebp-1c	XM_006532716.2	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Sirt-1	NM_001159289.2	GCAACAGCATCTTGCCTGAT	GTGCTACTGGTCTCACTT
Lpin-1	NM_001355598.1	CCATTCACAGCGAGTCTTCA	TGGAAGGGGAATCTGTCTTG
Actb	NM_007393.5	CCTCCCTGGAGAAGAGCTATG	TTACGGATGTCAACGTCACAC

Atgl, adipose triglyceride lipase; *Hsl*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *Cpt-1a*, carnitine palmitoyl transferase-1α; *Cd36*, cluster of differentiation 36; *FABPpm*, plasma membrane fatty acid

binding protein; *FATP-1*, fatty acid transport protein-1; *FATP-2*, fatty acid transport protein-2; *FATP-3*, fatty acid transport protein-3; *FATP-4*, fatty acid transport protein-4; *FATP-5*, fatty acid transport protein-5; *FATP-6*, fatty acid transport protein-6; *Fas*, fatty acid synthase; *Acc-1*, acetyl coenzyme A carbocylase-1; *Acc-2*, acetyl coenzyme A carbocylase-2; *Scd-1*, stearoyl-CoA desaturase-1; *Ppar-a*, peroxisome proliferator-activated receptor-α; *Srebp-1c*, sterol regulatory element binding protein-1c; *Sirt-1*, sirtuin-1; *Lpin-1*, lipin-1; *Actb*, β-actin.

2.2.7. Statistical Analysis

The results are expressed as mean ± standard error (SE). Statistical analyses were performed using the SPSS 24.0 statistical software package (SPSS Japan Inc., Tokyo, Japan). For inter-group comparisons of means and to confirm the normality in each group and each item, the Shapiro-Wilk test was performed. Unpaired *t*-tests were performed when both groups displayed a normal distribution and Mann-Whitney *U* tests were performed when either of the two groups did not display a normal distribution. The correlation between gene expression in various tissues/organs and plasma NEFA levels was assessed based on the Spearman's correlation coefficient (*r*). *P*-values less than 0.05 were considered statistically significant, whereas *P*-values less than 0.1 were considered to indicate a significant tendency. All statistical tests were two-tailed.

2.3. Results

2.3.1. Effects on Plasma Biochemical Data 30 Minutes After Transdermal Administration of Jojoba Oil

To examine the association between plasma biochemical data and the transdermal administration of jojoba oil, plasma lipid concentrations, including those of T-CHO, TG, PL, and NEFA, also known as free fatty acids (FFA), were measured. As shown in Table 4, plasma NEFA levels were significantly increased in comparison to those in the control

those in the control group ($p < 0.05$). In contrast, no significant differences were observed between the two groups in plasma T-CHO, TG, and PL. However, other biochemical parameters (ALB, BUN, CRE, etc.) remained unchanged. Together, these results indicate that 30 min after jojoba oil administration, plasma lipid levels increased because of increased NEFA.

Table 4. Comparison of plasma biochemical data in accordance with the presence or absence of jojoba oil transdermal administration.

		Control	Jojoba Oil	
ALB	(g/dL)	2.8 ± 0.1	2.9 ± 0.0	
BUN	(mg/dL)	25.9 ± 1.1	23.8 ± 1.0	
CRE	(mg/dL)	0.1 ± 0.0	0.1 ± 0.0	
UA	(mg/dL)	2.0 ± 0.2	2.2 ± 0.1	
AST	(IU/L)	99.5 ± 15.4	87.0 ± 6.8	
ALP	(IU/L)	401.7 ± 21.9	414.7 ± 17.0	
CK	(IU/L)	388.0 ± 102.0	216.7 ± 45.1	
T-CHO	(mg/dL)	73.8 ± 2.3	70.0 ± 1.9	
TG	(mg/dL)	67.5 ± 7.7	62.0 ± 6.7	
PL	(mg/dL)	154.3 ± 4.0	148.7 ± 5.5	
NEFA	(μEq/L)	626.2 ± 36.0	757.7 ± 22.8	*
LDL-C	(mg/dL)	4.8 ± 0.4	4.3 ± 0.6	
HDL-C	(mg/dL)	47.0 ± 1.5	44.3 ± 1.4	
TBA	(μmol/L)	1.2 ± 0.2	1.0 ± 0.0	
GLU	(mg/dL)	239.5 ± 17.5	223.7 ± 12.2	
LA	(mg/dL)	81.0 ± 6.9	66.8 ± 5.3	
T-KB	(μmol/L)	317.0 ± 25.5	338.7 ± 25.4	

ALB, albumin; BUN, blood urea nitrogen; CRE, creatinine; UA, uric acid; AST, asparatate aminotransferase; ALP, alkaline phosphatase; CK, creatine kinase; T-CHO, total cholesterol; TG, triglyceride; PL, phospholipid; NEFA, non-esterified fatty acids; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TBA, total bile acid; GLU, glucose; LA, lactic acid; T-KB, total ketone body. Values are means ± SE, n = 6. *$p < 0.05$.

2.3.2. Changes in Expression Levels of Lipid Metabolism-Related Genes in Various Tissues/Organs After Transdermal Administration of Jojoba Oil

To investigate the cause of increased plasma NEFA, real-time PCR were performed

to evaluate expression levels of the following in various tissues/organs: (1) lipid degradation-related genes; (2) fatty acid trafficking-related genes; (3) lipogenesis-related genes. The percentage values for changes in gene expression in various tissues/organs relative to those in the control group are shown in Table 5.

Table 5. Percentage values of changes in gene expression in various tissues/organs.

	Liver	WAT	Skin	BAT	Plantaris muscle	Heart
Lipid degradation						
Atgl	123.2 ± 8.0 *	138.4 ± 22.8	68.0 ± 3.6	102.5 ± 3.6	74.5 ± 8.2	92.4 ± 3.1
Hsl	95.0 ± 4.0	134.7 ± 21.3	55.4 ± 4.9 †	114.8 ± 2.9	84.3 ± 5.2	80.4 ± 9.1
Lpl	94.9 ± 3.8	135.7 ± 20.8	90.7 ± 15.4	99.0 ± 8.2	81.2 ± 6.1	90.0 ± 5.4
Cpt-1a	112.1 ± 6.8	112.9 ± 36.0	103.7 ± 8.2	89.6 ± 3.5	80.9 ± 10.6	99.3 ± 6.6
Fatty acid trafficking						
Cd36	140.4 ± 23.6	113.1 ± 16.4	108.6 ± 10.0	110.9 ± 3.5	94.6 ± 9.8	102.1 ± 6.4
FABPpm	101.6 ± 9.4	47.6 ± 34.4	64.7 ± 7.4 †	128.3 ± 34.2	88.5 ± 6.4	94.6 ± 6.2
FATP-1	83.0 ± 4.9	89.1 ± 16.3	30.0 ± 3.8 †	103.6 ± 6.6	61.0 ± 22.1	96.6 ± 3.4
FATP-2	112.6 ± 13.8	77.2 ± 52.1	N.D.	123.8 ± 11.6	87.2 ± 79.1	36.7 ±20.9
FATP-3	198.4 ± 21.5 **	100.8 ± 15.9	43.6 ± 4.8 †	84.2 ± 4.1	76.5 ± 7.0	140.7 ±30.1
FATP-4	80.8 ± 3.9 *	84.2 ± 26.3	69.0 ± 5.0 †	97.4 ± 4.9	76.3 ± 8.7	107.5 ±8.4
FATP-5	112.6 ± 11.0	53.3 ± 29.1	N.D.	39.7 ± 6.9	N.D.	106.6 ±36.7
FATP-6	N.D.	35.2 ± 15.5	N.D.	16.3 ± 4.8	65.5 ± 20.1	40.9 ± 14.5
Lipogenesis						
Fas	89.7 ± 9.9	99.3 ± 16.9	85.3 ± 4.0	87.5 ± 2.6 *	97.2 ± 12.5	51.1 ± 20.7
Acc-1	91.4 ± 6.6	119.7 ± 19.0	53.3 ± 3.6 †	87.6 ± 3.7 *	103.2 ± 5.8	58.7 ± 15.0
Acc-2	81.5 ± 6.6	138.0 ± 22.6	40.4 ± 3.4 *	100.2 ± 5.2	74.4 ± 5.4 *	91.5 ± 5.6
Scd-1	81.0 ± 7.1	133.4 ± 28.0	81.9 ± 16.9	96.0 ± 3.8	158.6 ± 28.5 †	81.7 ± 29.3
Nuclear transcrption factors						
Ppar-a	92.1 ± 8.3	125.6 ± 16.5	14.3 ± 4.2 *	129.8 ± 12.2	119.1 ± 44.0	85.9 ± 6.3
Srebp-1c	94.6 ± 7.7	95.5 ± 5.5	52.6 ± 9.4 **	87.2 ± 2.1 **	58.1 ± 2.6 *	78.0 ± 7.0
Sirt-1	157.6 ± 16.7 *	90.7 ± 9.7	127.1 ± 6.1 †	89.4 ± 2.6	116.2 ± 13.2	112.8 ± 7.2
Lpin-1	284.8 ± 68.6 *	130.5 ± 14.7	43.1 ± 5.0 *	95.5 ± 3.2	75.9 ± 7.6	76.4 ± 4.2

Atgl, adipose triglyceride lipase; *Hsl*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *Cpt-1a*, carnitine palmitoyl transferase-1α; *Cd36*, cluster of differentiation 36; *FABPpm*, plasma membrane fatty acid binding protein; *FATP-1*, fatty acid transport protein-1; *FATP-2*, fatty acid transport protein-2; *FATP-3*, fatty acid transport protein-3; *FATP-4*, fatty acid transport protein-4; *FATP-5*, fatty acid transport protein-5; *FATP-6*, fatty acid transport protein-6; *Fas*, fatty acid synthase; *Acc-1*, acetyl coenzyme A carbocylase-1; *Acc-2*, acetyl coenzyme A carbocylase-2; *Scd-1*, stearoyl-CoA desaturase-1; *Ppar-a*, peroxisome proliferator-activated receptor-α; *Srebp-1c*, sterol regulatory element binding protein-1c; *Sirt-1*, sirtuin-1; *Lpin-1*, lipin-1. N.D., not detected. Values are means ± SE, n = 6. $^{\dagger}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$.

(1) Changes in the Expression of Lipid Degradation-Related Genes

TGs are produced by the esterification of NEFA with glycerol, which is produced by sugar and alcohol metabolism. TGs are stored under the skin and around the viscera, and are metabolized into fatty acids, which serve as energy sources. Both ATGL and HSL enzymes are involved in TG degradation. *Atgl* was significantly upregulated in the liver 30 min after jojoba oil administration ($p < 0.05$), but there was no significant change seen in other tissues/organs. In contrast, there were no significant changes in *Hsl* expression in any tissues/organs 30 min after jojoba oil administration. Similarly, no changes were observed in the expression of *Lpl* which is involved in lipid uptake, and *Cpt-1a*, which regulates β-oxidation.

(2) Changes in the Expression of Fatty Acid Trafficking-Related Genes

Since fatty acids in the bloodstream are taken up by cells via CD36, FABPpm, and FATP family proteins, the expression levels of these genes were examined. *FATP-3* was significantly upregulated in the liver ($p < 0.05$) and *FATP-4* was significantly downregulated ($p < 0.05$). However, *FABPpm*, *FATP-1*, *FATP-3*, and *FATP-4* were downregulated in the skin of mice from the experimental group ($p = 0.067$, 0.074, 0.076, and 0.082, respectively).

(3) Changes in the Expression of Lipogenesis-Related Genes

To assess the expression levels of lipogenesis-related genes, the expression levels of *Fas*, *Acc-1*, *Acc-2*, and *Scd-1* were evaluated via real-time PCR analysis. *Fas* and *Acc-1* in the BAT were significantly downregulated in the experimental group ($p < 0.05$). Additionally, *Acc-2* in the skin and the plantaris muscle was significantly downregulated

in the experimental group ($p < 0.05$). In association with lipogenesis, *Srebp-1c*, coding a transcription factor associated with fatty acid synthesis by upregulating *Scd-1*, was significantly downregulated in the skin, BAT, and plantaris muscle ($p < 0.05$). However, *Scd-1* was not upregulated in any of these tissues/organs.

2.3.3. Correlation Between Lipid Metabolism-Related Genes Expressed in Various Tissues/Organs and Plasma NEFA Levels After Transdermal Administration of Jojoba Oil

To confirm that the alterations in gene expression levels in the associated tissues/organs were correlated with elevated plasma NEFA levels, we examined the relationship between gene expression levels in various tissues/organs and plasma NEFA levels (Table 6). The results of the analysis are described as follows for correlations among lipid degradation-related genes, fatty acid trafficking-related genes, lipogenesis-related genes, and plasma NEFA levels.

(1) Correlations Between Lipid Degradation-Related Genes Expressed in Various Tissues/Organs and Plasma NEFA Levels After Transdermal Administration of Jojoba Oil

As shown in Table 6 and Figure 1, liver *Atgl* expression levels were positively and significantly correlated with plasma NEFA levels ($r = 0.592$, $p < 0.05$), and skin *Hsl* expression levels tended to be negatively correlated with plasma NEFA levels ($r = -0.427$, $p = 0.083$).

Table 6. Associations among gene expression levels in various tissues/organs and plasma non-esterified fatty acid levels assessed on the basis of correlation coefficient.

	Liver	WAT	Skin	BAT	Planta	Heart
Lipid degradation						
Atgl	0.592 *	0.392	– 0.385	0.095	– 0.294	– 0.179
Hsl	– 0.178	0.025	– 0.427 †	0.235	– 0.179	0.196
Lpl	– 0.046	0.231	0.123	0.277	– 0.018	– 0.469
Cpt-1a	0.133	– 0.063	0.168	– 0.196	– 0.098	– 0.200
Fatty acid trafficking						
Cd36	0.528 †	– 0.035	– 0.032	0.179	– 0.287	– 0.263
FABPpm	0.049	– 0.081	– 0.133	0.182	– 0.266	– 0.375
FATP-1	0.305	0.028	– 0.284	0.238	– 0.203	– 0.298
FATP-2	0.098	0.091	N.D.	0.091	– 0.071	0.395
FATP-3	0.452	0.312	– 0.501 †	– 0.361	– 0.168	0.462
FATP-4	– 0.126	– 0.319	– 0.357	– 0.004	– 0.413	– 0.231
FATP-5	0.203	– 0.238	N.D.	– 0.056	N.D.	0.035
FATP-6	N.D.	– 0.207	N.D.	– 0.132	– 0.237	– 0.074
Lipogenesis						
Fas	– 0.084	– 0.004	0.070	– 0.228	0.294	0.487
Acc-1	0.025	0.277	– 0.147	– 0.137	0.025	0.347
Acc-2	0.700	0.362	– 0.280	0.109	– 0.333	– 0.392
Scd-1	0.105	0.259	– 0.123	0.155	0.490	0.581 *
Nuclear transcrption factors						
Ppar-a	– 0.123	0.028	– 0.452	0.109	0.053	– 0.424
Srebp-1c	– 0.389	– 0.119	– 0.438	– 0.497	– 0.567 †	– 0.291
Sirt-1	0.592 †	0.238	0.308	– 0.333	– 0.238	0.147
Lpin-1	0.583 †	0.228	– 0.543	0.130	– 0.158	– 0.609 *

Atgl, adipose triglyceride lipase; *Hsl*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *Cpt-1a*, carnitine palmitoyl transferase-1α; *Cd36*, cluster of differentiation 36; *FABPpm*, plasma membrane fatty acid binding protein; *FATP-1*, fatty acid transport protein-1; *FATP-2*, fatty acid transport protein-2; *FATP-3*, fatty acid transport protein-3; *FATP-4*, fatty acid transport protein-4; *FATP-5*, fatty acid transport protein-5; *FATP-6*, fatty acid transport protein-6; *Fas*, fatty acid synthase; *Acc-1*, acetyl coenzyme A carbocylase-1; *Acc-2*, acetyl coenzyme A carbocylase-2; *Scd-1*, stearoyl-CoA desaturase-1; *Ppar-a*, peroxisome proliferator-activated receptor-α; *Srebp-1c*, sterol regulatory element binding protein-1c; *Sirt-1*, sirtuin-1; *Lpin-1*, lipin-1; WAT, white adipose tissue; BAT, brown adipose tissue. N.D., not detected on gene expression level. Spearman's correlation coefficients of plasma NEFA levels with the expression levels of lipid metabolism-related genes. †$p < 0.1$, *$p < 0.05$.

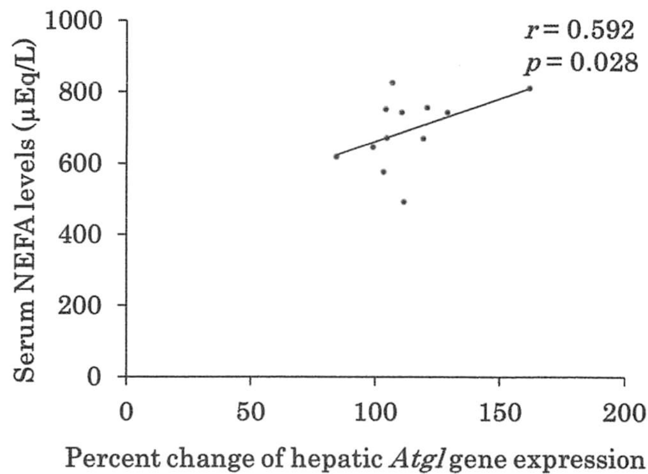


Figure 1. Correlation between plasma non-esterified fatty acid (NEFA) levels and liver *Atgl* expression levels at 30 min after topical application of jojoba oil. Plasma NEFA levels were significantly correlated with liver *Atgl* expression levels ($r = 0.592$, $p = 0.028$).

(2) Correlations Between Fatty Acid Trafficking-Related Genes Expressed in Various Tissues/Organs and Plasma NEFA Levels After Transdermal Administration of Jojoba Oil

Liver *Cd36* expression levels tended to be positively correlated with plasma NEFA levels ($r = 0.528$, $p = 0.095$), whereas skin *FATP-3* expression levels tended to be negatively correlated with plasma NEFA levels ($r = -0.501$, $p = 0.097$).

(3) Correlations Between Lipogenesis-Related Genes Expressed in Various Tissues/Organs and Plasma NEFA Levels After Transdermal Administration of Jojoba Oil

Cardiac *Scd-1* expression levels were positively and significantly correlated with plasma NEFA levels ($r = 0.581$, $p < 0.05$).

(4) Correlations Between Nuclear Transcription Factors Expressed in Various Tissues/Organs and Plasma NEFA Levels After Transdermal Administration of Jojoba Oil

Cardiac *Lpin-1* expression levels were negatively and significantly correlated with plasma NEFA levels (r = −0.609, p < 0.05). Furthermore, liver *Sirt-1* and *Lpin-1* expression levels tended to be positively correlated with plasma NEFA levels (r = 0.592, p = 0.055; r = 0.583, p = 0.060, respectively), whereas *Srebp-1c* expression levels in the plantaris muscle tended to be negatively correlated with plasma NEFA levels (r = −0.567, p = 0.054).

2.4. Discussion

2.4.1. Potential Mechanisms Underlying Elevated Plasma NEFA Levels

The results from the present study show that plasma NEFA levels are elevated upon topical administration of jojoba oil in mice. Jojoba oil has anti-inflammatory effects, anti-skin-aging effects, wound healing effects, antioxidant effects, antibacterial effects, and antifungal effects (55, 69-71). However, no studies have reported the effects of transdermal application of jojoba oil on lipid metabolism. To our knowledge, this is the first report on the analysis of plasma biochemical data after transdermal administration of jojoba oil and gene expression analysis of lipid metabolism-related genes in various tissues/organs. In the present study, plasma NEFA levels were significantly increased 30 min after topical application of jojoba oil (Table 4).

The potential mechanisms underlying the elevation in plasma NEFA levels are described as follows. As previously indicated, jojoba oil is composed of a liquid wax ester, which is an ester comprised of fatty acids (73.4% eicosenoic acid, 14.7% erucic acid, and

8.3% oleic acid) and fatty alcohols. Thus, this study presume that these fatty acids are absorbed into the skin and, subsequently, modify the expression of transcription factors and genes correlated with fatty acid metabolism, through a mechanism of substrate-dependent gene expression.

Since NEFA levels were increased in the jojoba oil-treated group based on an examination of plasma biochemistry, the relative expression of lipid metabolism-related genes was analyzed. NEFA is rapidly metabolized in the blood and has a half-life for 1–2 min. The following events might elevate plasma NEFA levels: (1) TG degradation in the WAT, and decomposition of intrahepatic TG droplets into fatty acids and glycerol; (2) decreased intracellular uptake of fatty acids into cells by CD36, FABPpm, and the FATP family; (3) a fatty acid supply to blood via lipogenesis. Therefore, real-time PCR were performed to evaluate the expression levels in various tissues/organs of (1) lipid degradation-related genes, (2) fatty acid trafficking-related genes, and (3) lipogenesis-related genes.

Since TG degradation involves both ATGL and HSL enzymes, the gene expression levels of both *Atgl* and *Hsl* were analyzed in this study. Regarding lipolysis-related gene expression, *Atgl* was upregulated only in the liver, and no change was observed in the WAT, skin, BAT, plantaris muscle, and heart (Table 5). Furthermore, a significant positive correlation was observed between the relative expression levels of *Atgl* in the liver, and plasma NEFA levels after topical application of jojoba oil (Figure 1). Therefore, degradation of lipid droplets in the liver may contribute to the increase in plasma NEFA levels after this treatment. Because ATGL is a triacylglycerol hydrolase that promotes the lipolysis of stored fat (72), an association between the upregulation of *Atgl* in the liver and elevated plasma NEFA levels indicates that hepatic lipolysis caused the release

of NEFA into the blood, and increased plasma NEFA levels.

Since fatty acids in the bloodstream are taken up by cells via CD36, FABPpm, and the FATP family proteins, the expression of these genes was examined. As a result, after 30 min of topical application of jojoba oil, *FABPpm*, *FATP-1*, *FATP-3*, and *FATP-4* tended to be downregulated in the skin. The reason for this might be related to SREBP-1, which regulates the expression levels of *Lpin-1*. Lipin 1 is a co-factor required for PPAR-α activation (73), and PPAR-α regulates *FATP* mRNA expression (74). Therefore, the significant decrease in expression levels of *Srebp-1c*, *Lpin-1*, and *Ppar-a* in the skin could be related to the fact that the expression levels of fatty acid trafficking-related genes also tended to be downregulated in the skin. Considering that the skin is the largest organ, the decreased expression of four fatty acid trafficking-related genes in the skin might have potentially increased plasma NEFA levels. In this study, skin *FATP-2*, *FATP-5*, and *FATP-6* expression levels were considered at the lower limit of determination. These results are concurrent with those of previous studies (75). In the liver, although *FATP-3* was significantly upregulated, *FATP-4* was significantly downregulated. However, Schaffer reported that *FATP* mRNA is highly expressed in skeletal muscle, the heart, and body fat, but found at low levels in the livers of normal mice (76). Therefore, hepatic lipid intake might not be affected, when taking into account the offset of increased hepatic *FATP-3* expression levels by decreased hepatic *FATP-4* expression levels.

Regarding lipogenesis-related gene expression, Acetyl-CoA carboxylase (ACC-1, ACC-2) promotes fatty acid synthesis. In addition, fatty acid synthase (FAS) is an important rate-limiting step in lipogenesis. ACC catalyzes the conversion of acetyl CoA to malonyl-CoA, which is a potent inhibitor of carnitine palmitoyl transferase-1 (CPT-1). Therefore, ACC-2 indirectly prevents the influx of fatty acids into the mitochondria and

subsequent β-oxidation (77). Thus, it is possible that β-oxidation of mitochondrial fatty acid increased after 30 min, subsequent to jojoba oil administration in the skin and plantaris muscle, where *Acc-2* gene expression was significantly reduced. Since SREBP-1c is a master regulator of lipogenesis (78, 79), the reason for the significantly downregulated expression levels of lipogenesis-related genes in the skin, BAT, and plantaris muscle could be related to downregulated expression of *Srebp-1c*.

2.4.2. Potential Clinical Applications of Jojoba Oil and Future Prospects

To date, several studies have examined the effects of vegetable oils, in the presence or absence of massage, on neonates, and reported that neonatal growth is promoted through oil massages (80-83). Although the mechanisms of action are yet unclear, triglycerides and fatty acids are suggested to be significantly elevated upon topical application of vegetable oils, which may potentially serve as a nutrient source and, hence, promote neonatal growth. Moreover, some studies have suggested that topical application of vegetable oils is significantly associated with changes in vagal tone during massaging, and changes in gastric motility after a massage (84). Unlike TG-based vegetable oils, jojoba oil used in this study is based on wax esters. However, the significant increase in fatty acids after topical application is a feature common to both jojoba and vegetable oils.

The significant increase in plasma fatty acid levels after topical application of jojoba oil provides novel insights into the potential applications of this oil to help promote the growth of neonates, improve nutrition among elderly individuals, improve endurance exercises among athletes, and improve the therapeutic effects of physical therapy. Further studies are needed to clarify whether the plasma fatty acids, which increased

upon topical application of jojoba oil, are used as energy substrates.

This study has potential limitations. First, the dose of oil administered to the mice was larger than the dose usually administered to humans for massage or rubbing (equivalent to 240 mL of oil for a 60-kg person). However, massage and rubbing could enhance lipid absorption. Since massage or rubbing increases blood flow and skin temperature, it has been reported that these techniques are likely to alter the stratum corneum structure and enhance transdermal absorption (85). Second, the small sample number could hinder the detection of some slight differences. Furthermore, the skin has numerous functions. However, the most obvious function is both a physical and a biological defense. Therefore, not all compounds can penetrate the skin, and a characteristic molecular weight of 500 Da or less is important for transdermal absorption (86). As shown in Table 1, the molecular weights of fatty acids contained in jojoba oil are less than 500 Da. However, fatty acids contained in jojoba oil are present as wax esters, and it remains unclear whether wax esters are degraded into fatty acids and alcohols by the skin microflora. Moreover, according to confocal microscopic studies examining the penetration of jojoba oil into human skin, jojoba oil penetrates only into the outermost layers of the stratum corneum (57, 58, 87). Therefore, it is an open question whether the fatty acid constituting the wax ester, per se, are percutaneously absorbed into the blood. Although certain aspects remain unknown, it is clear that specific constituents of jojoba oil penetrated the skin after topical application.

2.5. Conclusions

This study shows that plasma NEFA levels are elevated 30 min after transdermal administration of jojoba oil, thus indicating that certain constituents of jojoba oil

penetrate the skin. The mechanism underlying elevated plasma NEFA levels might be comprised of both enhanced lipolysis via *Atgl* upregulation in the liver, and reduced fatty acid trafficking via *FABPpm*, *FATP1*, *FATP3*, and *FATP4* downregulation in the skin. Further studies are needed to clarify what constituents of jojoba oil can be absorbed transdermally.

Chapter 3.

Effects of Topical Application of Jojoba Oil on Exercise-Induced Inflammation

3.1. Introduction

Regular exercise reduces the risk of many diseases, such as hypertension, stroke, coronary heart disease, cancer, type 2 diabetes mellitus, dyslipidemia, obesity, osteoporosis, and depression (88-90). In contrast, exercise-induced muscle damage and delayed-onset muscle soreness (DOMS) are caused by exhaustive exercise (3) and are characterized by secondary inflammation and pain resulting from leukocyte infiltration into the damaged muscle (7, 91, 92). DOMS causes muscle weakness, swelling, and reduced range of motion (4, 5). It usually occurs within the first 24 h after exhaustive exercise and peaks between 24 and 72 h (92). Therefore, relieving the symptoms of muscle damage and inflammation might be beneficial to individuals who require rapid recovery in the short term (93, 94).

In recent years, the usefulness of sports massage for alleviating exercise-induced inflammation (24) and DOMS (21, 59, 95) and improving muscle performance has been reported (4, 59). It is believed that massage can be used to passively move and stimulate muscles to promote the circulation of blood and lymph, promote metabolism in muscles, and eliminate muscle fatigue. In Europe, the birthplace of massage, sports massage is performed using various types of massage oils, such as jojoba oil and sweet almond oil. By using these oils, the friction between the skin and the hands can be reduced, and the

massage can be performed smoothly. Jojoba oil is also used as carrier oil when diluting essential oils. Although there is extensive research on massage therapy, information on the biological effects of carrier oils is limited. In addition, some review articles have not indicated the type of massage oils used in previous studies (27, 54, 59-63). Researchers and therapists seem to consider the biological effects of carrier oils used to dilute essential oils negligible. However, jojoba oil, which is used in sports massage, reportedly exerts anti-inflammatory effects against carrageenin-induced rat paw edema and croton oil-induced ear edema (55, 96). Therefore, this study focused on the possibility that jojoba oil applied before exercise could prevent exercise-induced inflammation associated with muscle damage.

Meanwhile, it is known that lipid utilization increases during endurance exercise (97). Recently, it was revealed that plasma non-esterified fatty acid (NEFA) levels were increased by approximately 20%, 30 min after the topical application of jojoba oil in mice (98). When plasma NEFA is used as an energy source in skeletal muscle, it might affect run time-to-exhaustion during treadmill running. Therefore, the purpose of the present study was to investigate whether topical application of jojoba oil prior to treadmill running prevents exercise-induced muscle damage and whether it affects run time-to-exhaustion.

3.2. Materials and Methods

3.2.1. Ethics Statement

All animals were cared for in accordance with Law No. 105 and Notification No. 6 of the Japanese Government, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Waseda University (2019-A132).

3.2.2. Animals

Seven-week-old male hairless mice (Hos-HR-1) were purchased from Hoshino Laboratory Animals, Inc. (Bando, Japan) through Japan SLC, Inc. (Hamamatsu, Japan) and housed in a breeding room for at least 1 week. All mice were 8 weeks of age (28.2–34.8 g; average = 31.1 g) at the beginning of the experiments. The mice were housed under specific pathogen-free conditions with a standard commercial diet (MF, Oriental yeast, Tokyo, Japan), and water was provided *ad libitum*. The mice were housed under specific pathogen-free conditions at 22–24°C with 50–60% relative humidity and a light-dark cycle (lights on at 08:00 and off at 20:00).

3.2.3. Chemicals

Jojoba oil was obtained from Kenso-Igakusha (Yamanashi, Japan; Lot. GL15A). The ingredients of jojoba oil used in this study are listed according to the manufacturer's package insert (Table 7). Jojoba oil, a fatty acid-containing wax ester, is different from common vegetable oils rich in triglycerides (TGs).

Table 7. Fatty acid composition of jojoba oil.

Name	MW [g/mol]	Content [%]
Eicosenoic acid	310.51	73.4
Erucic acid	338.57	14.7
Oleic acid	282.47	8.3

MW, molecular weight

3.2.4. Experimental Protocol

One week before exhaustive exercise, all exercise group mice were acclimated to running on a motorized treadmill at 0% grade and at a speed of 15 m/min for 10 min. On the day of the experiment, all mice were randomly assigned to one of the four groups

containing seven animals each as follows: the naïve control group (NA); the sedentary group with jojoba oil applied (JO); the exercise group with no oil applied (NA + Ex); and the exercise group with jojoba oil applied (JO + Ex). Based on the results of previous studies (67, 68, 98), the mice were topically administered 4 μL of jojoba oil per gram of body weight to the dorsal skin 30 min before treadmill running. Endurance exercise was performed on a motorized treadmill (Natsume Seisakusyo Co., Ltd., Tokyo, Japan), and the run time-to-exhaustion was recorded. Mice in the NA + Ex and JO + Ex groups were subjected to treadmill running at 10 m/min for 15 min, followed by 15 m/min and 20 m/min for 15 min each and then 24 m/min at a constant 7% grade until exhaustion. The sedentary group was made to fast while the exercise group was running, in order to adjust feeding conditions. Exhaustion was defined as the inability to continue regular treadmill running despite being stimulated via repeated gentle tapping on the back with a silicon rubber spatula. The endpoint of exhaustion for all of the run time-to-exhaustion tests within a study was determined by a treatment-blinded single observer to minimize observer bias. Electric shock was not used in order to avoid stressing mice. This method, which is a humane improvement and reduces the confounding effect of negative reinforcement on blood cytokine levels caused by the use of shock grids (99). Immediately after exhaustion, blood samples were drawn from the heart under light anesthesia via an inhalant isoflurane (Abbott, Tokyo, Japan) and placed in heparin tubes, following which isolated muscle tissues were immediately frozen in liquid nitrogen. Plasma, obtained from blood samples by centrifugation at 1,500 × *g* for 10 min at 4°C, were stored at −80°C until analysis.

3.2.5. Biochemical Analysis of Plasma

Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase

(AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), creatine kinase (CK), TG, NEFA, glucose (GLU), and lactic acid (LA) were measured at Kotobiken Medical Laboratories (Tsukuba, Japan). The plasma levels of ALT and AST were determined by the JSCC transferable method using L-Type ALT J2 and L-Type AST J2, respectively (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The plasma ALP level was measured by the IFCC transferable method using L-Type ALP IFCC (FUJIFILM Wako Pure Chemical Corporation). The plasma level of BUN was determined based on the urease-GlDH method using L-Type UN·V (FUJIFILM Wako Pure Chemical Corporation). The plasma CRE level was determined according to an enzymatic method using Determiner L CRE (Hitachi Chemical Diagnostics Systems Co., Ltd., Tokyo, Japan). The plasma level of CK was analyzed via the JSCC transferable method using L-Type CK (FUJIFILM Wako Pure Chemical Corporation). The plasma levels of TG and NEFA were determined based on an enzymatic method using Determiner C-TG (Hitachi Chemical Diagnostics Systems Co.) and NEFA-HR II (FUJIFILM Wako Pure Chemical Corporation), respectively. The plasma GLU level was measured by the hexokinase-G-6-PDH method using L-Type Glu 2 (FUJIFILM Wako Pure Chemical Corporation) with a BioMajesty™ automated analyzer (JCA-BM9130; JEOL Ltd., Tokyo, Japan). The plasma level of LA was determined according to an enzymatic method using Determiner LA (Hitachi Chemical Diagnostics Systems Co.). All measurements, except for GLU, were quantified using a BioMajesty™ autoanalyzer (JCA-BM8060; JEOL Ltd.).

3.2.6. RNA Isolation and Gene Expression Analysis Using Real-Time Polymerase Chain Reaction (Real-Time PCR)

Tissue samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed. Total RNA from the soleus muscle (slow-twitch muscle), the gastrocnemius muscle (fast-twitch muscle), the heart, and the liver was extracted using acid guanidinium thiocyanate-phenol-chloroform extraction (100, 101) combined with the bead homogenization method using a Shake Maser Neo (Bio Medical Science, Tokyo, Japan). The purity and quality of the total RNA from each sample were assessed based on the 260:280 nm optical density ratio, using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA). Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA), in accordance with the manufacturer's instructions. PCR was conducted via the StepOnePlus™ system (Applied Biosystems), using the Fast SYBR® Green Master Mix (Applied Biosystems). Cycling conditions were as follows: 10 s at 95°C for denaturation, followed by 45 cycles at 95°C for 5 s, 57°C for 10 s (annealing), and lastly, 72°C for 10 s. Data were normalized to *β-actin* levels as an internal standard, using the calibration curve method. The genes and primers used are listed in Table 8.

Table 8. Primers used for real-time polymerase chain reaction analysis.

Gene	Accession no.	Forward	Reverse
Il-1b	NM_008361.4	TGCCACCTTTTGACAGTGATG	TGTGCTGCTGCGAGATTTGA
Il-1ra	NM_031167.5	TGTGCCAAGTCTGGAGATGA	TTCTTTGTTCTTGCTCAGATCAGT
Il-6	NM_001314054.1	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
Il-10	NM_010548.2	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
iNOS	NM_001313922.1	GGGCTGTCACGGAGATCA	CCATGATGGTCACATTCTGC
Actb	NM_007393.5	CCTCCCTGGAGAAGAGCTATG	TTACGGATGTCAACGTCACAC

Il-1b, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase; *Actb*, β-actin.

3.2.7. Quantitative Analysis of Inflammation-Related Plasma Cytokines

Plasma levels of granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), interferon (IFN)-β, IFN-γ, interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, and tumor necrosis factor (TNF)-α were measured using the LEGENDplex™ Mouse Inflammation Panel (Lot No. B298993; Cat no. 740150; BioLegend, San Diego, CA, USA) in accordance with the manufacturer's instructions. The analysis was performed via a BD LSRFortessa Special Order Research Product analytical cytometer (Becton Dickinson, San Jose, CA, USA) and data were evaluated using the LEGENDplex™ Data Analysis software.

3.2.8. Statistical Analysis

Values are presented as mean ± standard error (SE). For comparisons between two groups, the Student's unpaired *t*-test was used. Before using the Student's unpaired *t*-test, the Shapiro-Wilk test was performed to confirm whether the data satisfied the normality assumption. For comparison between the means of four groups, one-way analysis of variance and Tukey's *post-hoc* test were performed. Correlations were determined using Spearman's correlation analysis. Statistical analyses were performed using the SPSS 24.0 software package (IBM Japan Inc., Tokyo, Japan). Statistical significance was set as $p < 0.05$.

3.3. Results

3.3.1. Effect of Topical Application of Undiluted Jojoba Oil Before Exercise on Endurance Performance

As shown in Figure 2, there was no statistical difference between the two groups

in run time-to-exhaustion ($p = 0.175$). The run time-to-exhaustion of the NA + Ex group and JO + Ex groups was 225 ± 23 min and 185 ± 10 min, respectively.

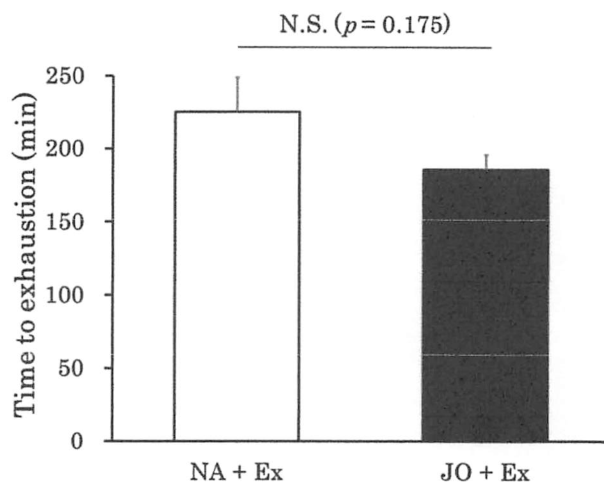
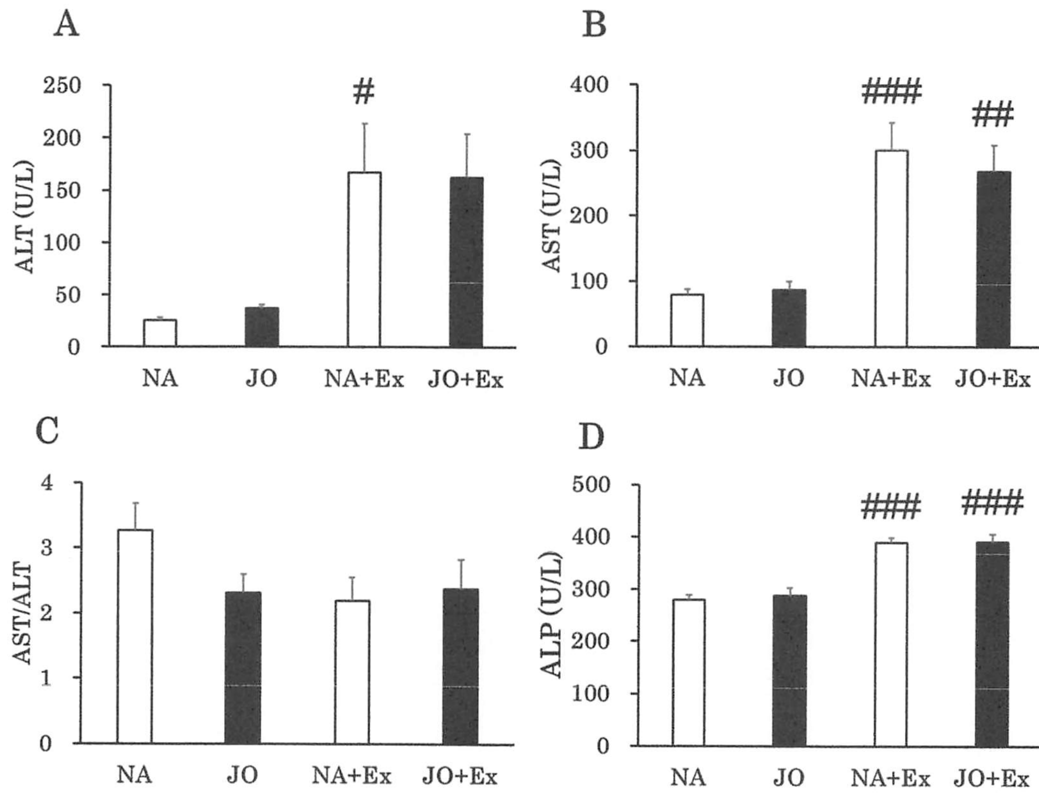


Figure 2. Run time-to-exhaustion in the exercise group with no oil applied (NA + Ex, n = 7) and the exercise group treated with jojoba oil (JO + Ex, n = 7). Values are presented as the mean ± standard error (SE). N.S., not significant.

3.3.2. Biochemical Analysis of Plasma

Plasma biochemistry results for the sedentary and exercise groups in the absence or presence of jojoba oil are shown (Figures 3 A-L). The plasma levels of ALT, AST, ALP, BUN, BUN/CRE, and CK were significantly increased in the NA + Ex group as compared to NA group, whereas the plasma levels of NEFA, GLU, and LA were significantly decreased. Similarly, the plasma levels of AST, ALP, BUN, BUN/CRE, and CK were increased in the JO + Ex group as compared to JO group. These differences were statistically significant for AST, ALP, BUN, and BUN/CRE and nearly significant for CK ($p = 0.058$). The plasma levels of GLU and LA were decreased; these differences were statistically significant for GLU and nearly significant for LA ($p = 0.058$). The only parameter that was significantly different in the sedentary group was the plasma levels

of NEFA; that is, the plasma levels of NEFA were significantly lower in the JO group than in the NA group. In contrast, changes in various parameters due to the application of jojoba oil were not observed between the two exercise groups (NA + Ex and JO + Ex).



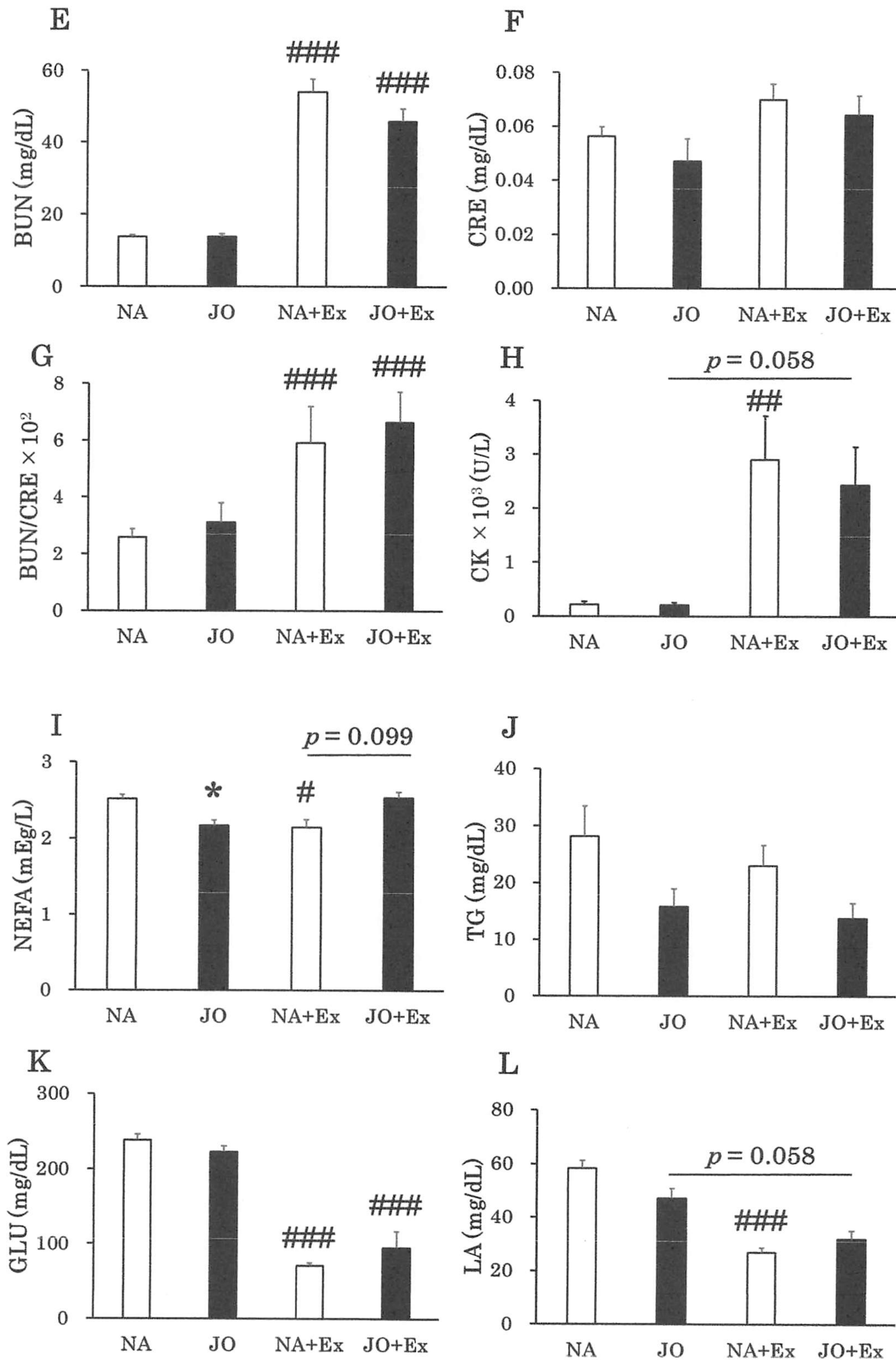


Figure 3. Plasma levels of (A) ALT, (B) AST, (C) AST/ALT, (D) ALP, (E) BUN, (F) CRE, (G) BUN/CRE, (H) CK, (I) NEFA, (J) TG, (K) GLU, and (L) LA. In the exercise groups (NA + Ex, JO + Ex), blood samples

were taken from the heart immediately after exhaustion. In the sedentary groups (NA, JO), blood samples were taken at the same time as that of the exercise groups for comparison. While mice in the exercise groups were running, those in the sedentary groups were fasting to adjust feeding conditions. NA, the naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRE, creatinine; CK, creatine kinase; TG, triglyceride; NEFA, non-esterified fatty acids; GLU, glucose; LA, lactic acid. Values are means ± SE, n = 7. $^{*}p < 0.05$ as compared to the NA group. $^{\#}p < 0.05$ as compared to the corresponding sedentary group. $^{\#\#}p < 0.01$ as compared to the corresponding sedentary group. $^{\#\#\#}p < 0.001$ as compared to the corresponding sedentary group.

3.3.3. Effects of Exhaustive Exercise With or Without Topical Application of Jojoba Oil on the Expression Levels of Inflammation-Related Genes in the Soleus Muscle

This study measured the inflammation-related mRNA expression levels in various organs/tissues to assess the anti-inflammatory effect of the topical application of jojoba oil on treadmill running in mice. In the soleus muscle, the expression levels of *Il-1b*, *Il-6*, and *Il-10* genes were significantly increased by exhaustive exercise (Figure 4). However, the JO + Ex group did not show a significant increase in the expression levels of these genes after treadmill running. In the exercise groups, the gene expression levels of *Il-6* and *Il-10* were significantly lower in the JO + Ex group than in the NA + Ex group. Moreover, the expression levels of *iNOS* were significantly decreased in both groups due to exercise. However, there were no significant differences in *Il-1ra* expression levels among the four groups. In addition, in the comparison between the sedentary groups, application of jojoba oil caused no changes in the expression levels of the inflammation-related genes.

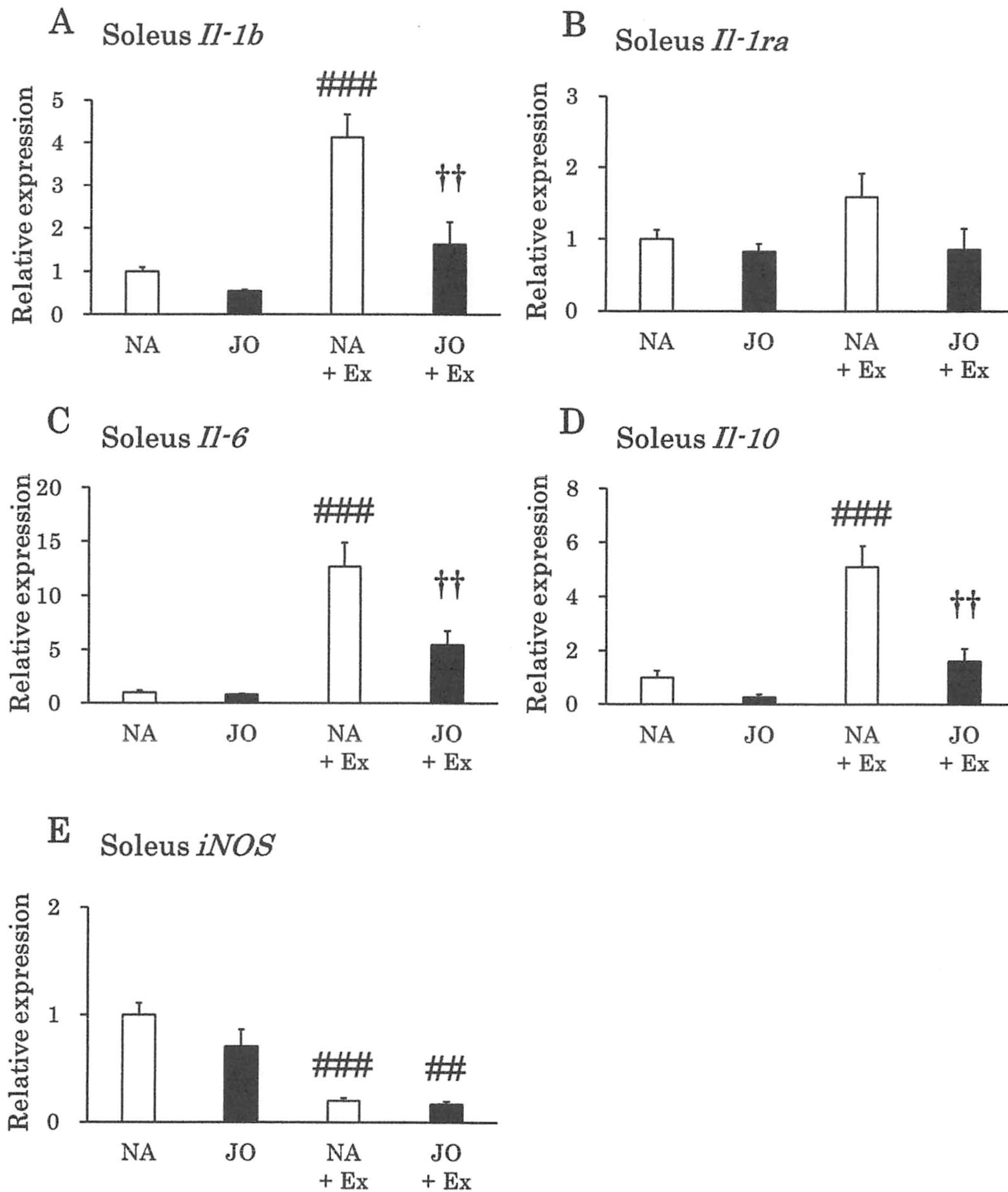
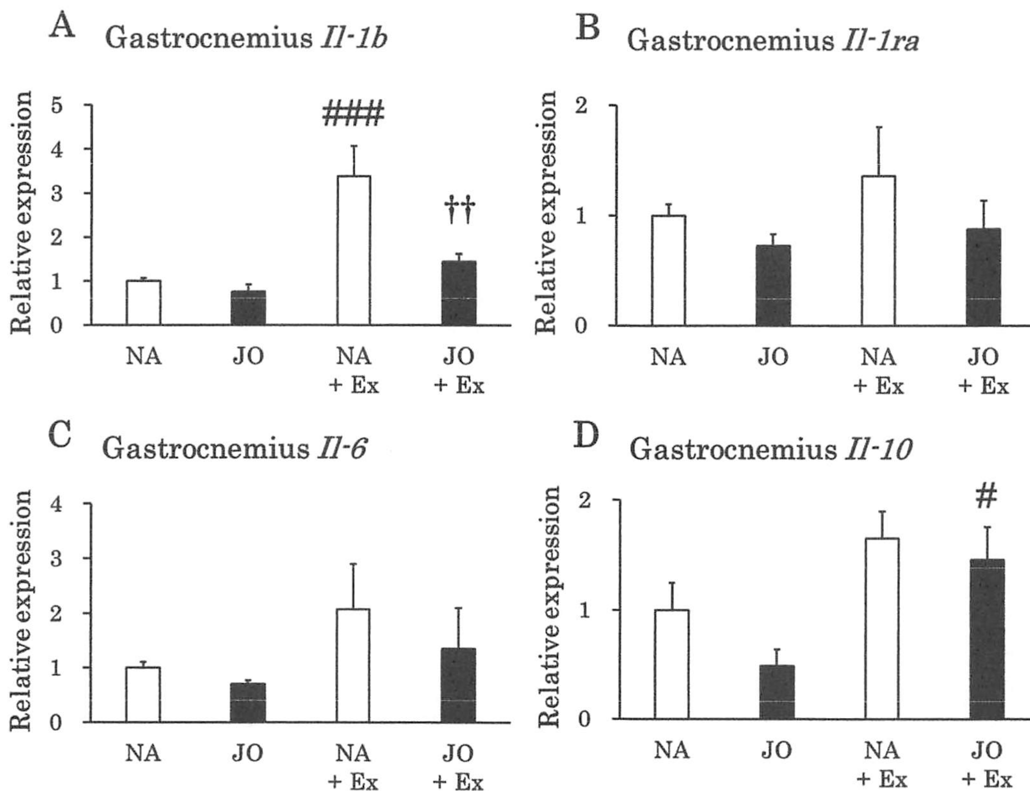


Figure 4. Gene expression levels of inflammation-related cytokines and *iNOS* in the soleus muscle. NA, the naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 7. ##$p < 0.01$ as compared to the corresponding sedentary group. ###$p < 0.001$ as compared to the corresponding sedentary group. ††$p < 0.01$ as compared to the NA + Ex group.

3.3.4. Effects of Exhaustive Exercise With or Without Topical Application of Jojoba Oil on the Expression Levels of Inflammation-Related Genes in the Gastrocnemius Muscle

The expression level of *Il-1b* in the gastrocnemius muscle was significantly increased by exhaustive exercise (Figure 5). However, the expression level of *Il-1b* in the JO + Ex group was significantly lower than that in the NA + Ex group. The expression levels of *Il-10* were significantly increased by exercise after the administration of jojoba oil. Furthermore, the expression levels of *iNOS* were significantly decreased in the NA + Ex group as compared to those in the NA group. There were no significant differences in *Il-6* and *Il-1ra* expression levels among the four groups. In addition, in the comparison between the sedentary groups, application of jojoba oil caused no changes in the expression levels of the inflammation-related genes.



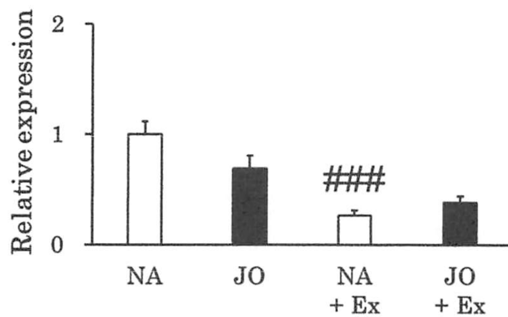


Figure 5. Gene expression levels of inflammation-related cytokines and *iNOS* in the gastrocnemius muscle. NA, the naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 7. $^{\#}p < 0.05$ as compared to the corresponding sedentary group. $^{\#\#\#}p < 0.001$ as compared to the corresponding sedentary group. $^{\dagger\dagger}p < 0.01$ as compared to the NA + Ex group.

3.3.5. Effects of Exhaustive Exercise With or Without Topical Application of Jojoba Oil on the Expression Levels of Inflammation-Related Genes in the Heart

The expression levels of *Il-10* in the heart were significantly increased by exhaustive exercise, whereas *iNOS* expression levels were significantly decreased (Figure 6). In contrast, there were no significant differences in *Il-1b*, *Il-1ra*, and *Il-6* expression levels in the heart among the four groups.

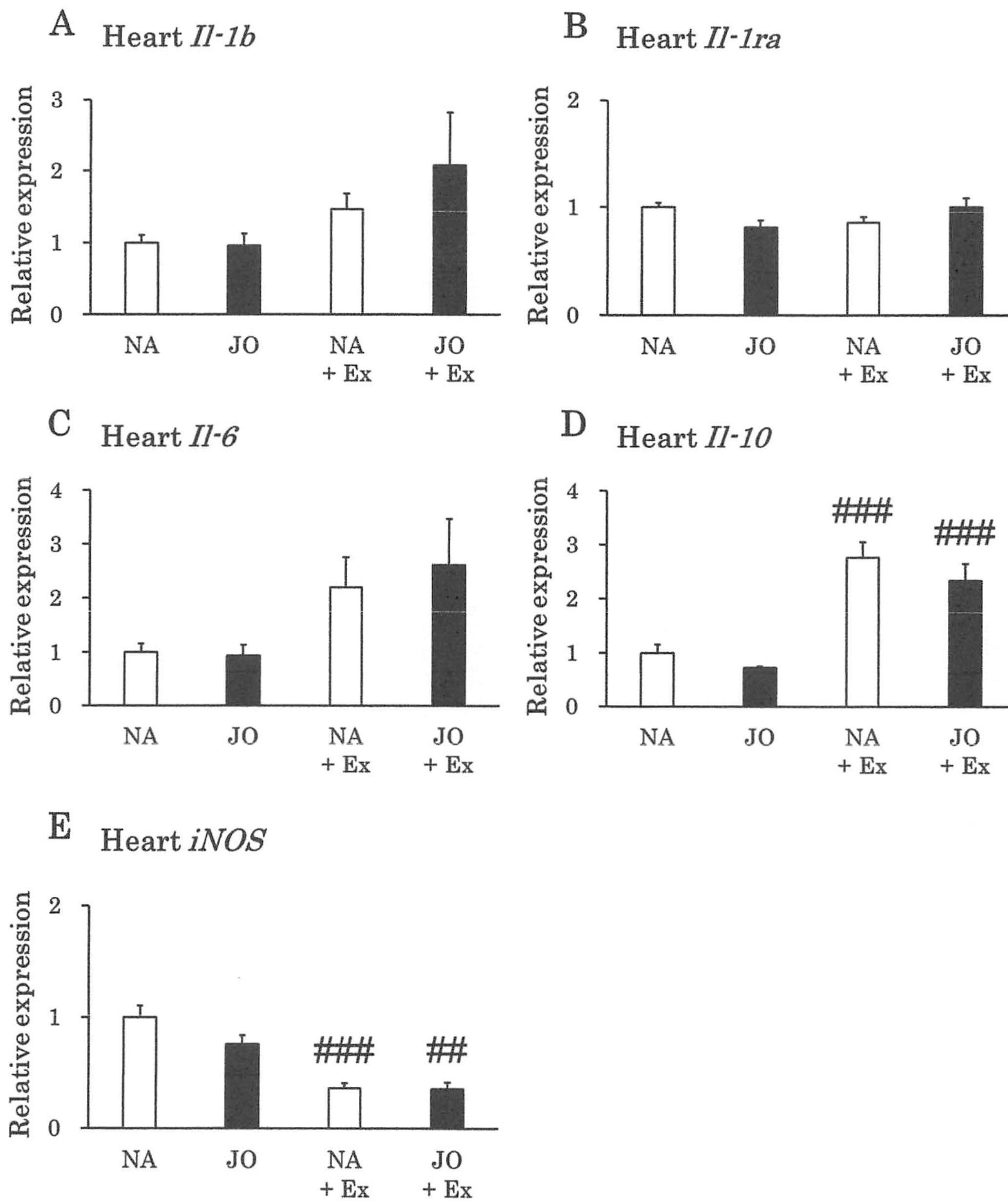
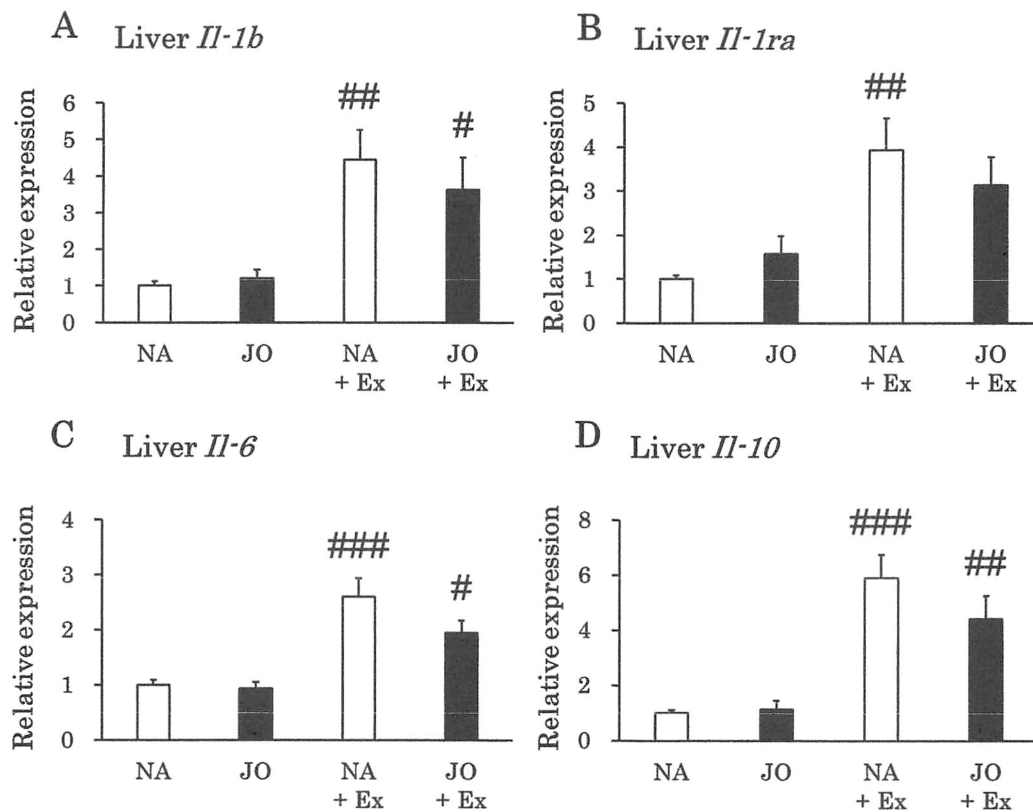


Figure 6. Gene expression levels of inflammation-related cytokines and *iNOS* in the heart. NA, the naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 7. ##$p < 0.01$ as compared to the corresponding sedentary group. ###$p < 0.001$ as compared to the corresponding sedentary group.

3.3.6. Effects of Exhaustive Exercise With or Without Topical Application of Jojoba Oil on the Expression Levels of Inflammation-Related Genes in the Liver

The expression levels of *Il-1b*, *Il-6*, and *Il-10* in the liver were significantly increased by exhaustive exercise (Figure 7). The expression level of the *Il-1ra* gene was also increased by exercise, but only in the jojoba oil-applied group. In contrast, the expression levels of *iNOS* were significantly decreased only in the NA + Ex group by exhaustive exercise.



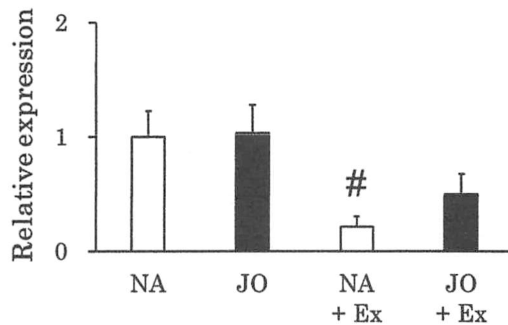
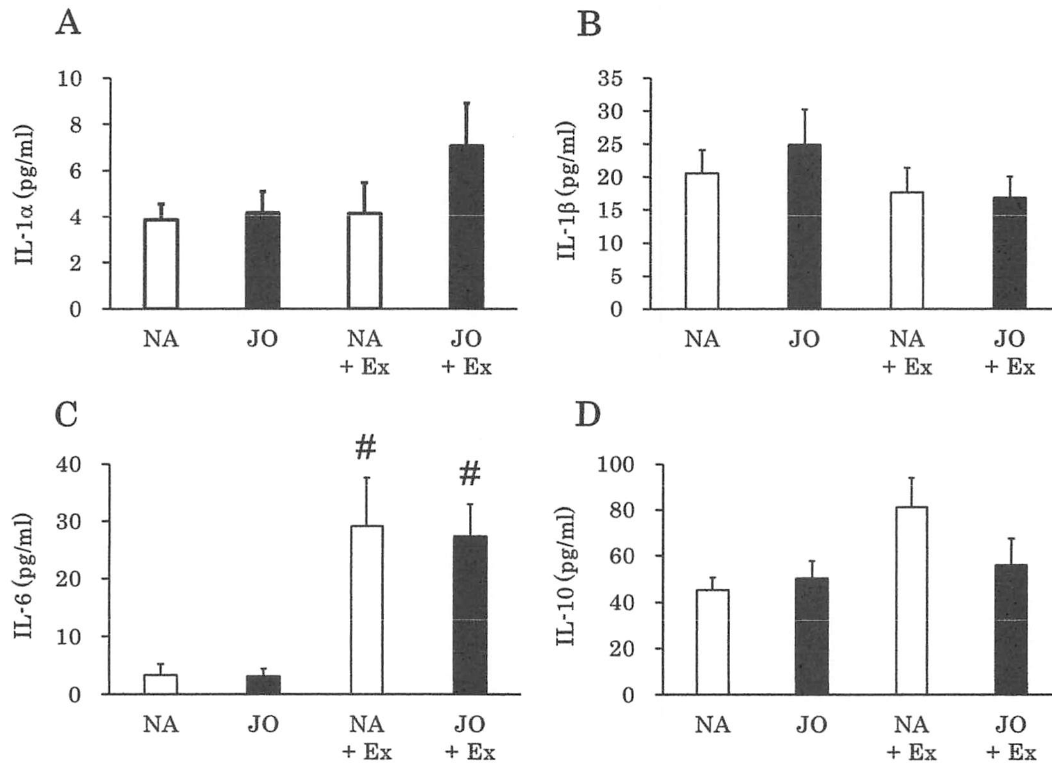


Figure 7. Gene expression levels of inflammation-related cytokines and *iNOS* in the liver. NA, the naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 7. $^{\#}p < 0.05$ as compared to the corresponding sedentary group. $^{\#\#}p < 0.01$ as compared to the corresponding sedentary group. $^{\#\#\#}p < 0.001$ as compared to the corresponding sedentary group.

3.3.7. Quantitative Analysis of Inflammation-Related Plasma Cytokines Levels

In the exercise groups, the gene expression levels of inflammatory cytokines in the soleus and gastrocnemius muscles were significantly reduced by the application of jojoba oil. Therefore, as shown in Figure 8, plasma cytokine levels were quantified by flow cytometry to confirm whether systemic cytokines were altered in response to the topical application of jojoba oil. The plasma levels of IL-1α, IL-1β, IL-10, IL-17A, IL-27, IFN-β, GM-CSF, and MCP-1 did not show significant changes among all groups. In contrast, the plasma levels of IL-6 were significantly elevated by exhaustive exercise in the NA + Ex and JO + Ex groups, whereas the plasma levels of TNF-α were significantly decreased in these groups. In addition, with respect to the sedentary groups, the plasma levels of IFN-γ were significantly decreased in the JO group as compared to those in the NA group and significantly decreased in the NA + Ex group compared to those in the NA group.

However, for the exercise groups, there were no significant changes between the NA + Ex and JO + Ex groups for all cytokines measured, and the plasma levels of IL-12p70 and IL-23 were lower than the limit of detection (2.54 pg/ml and 33.09 pg/ml, respectively).



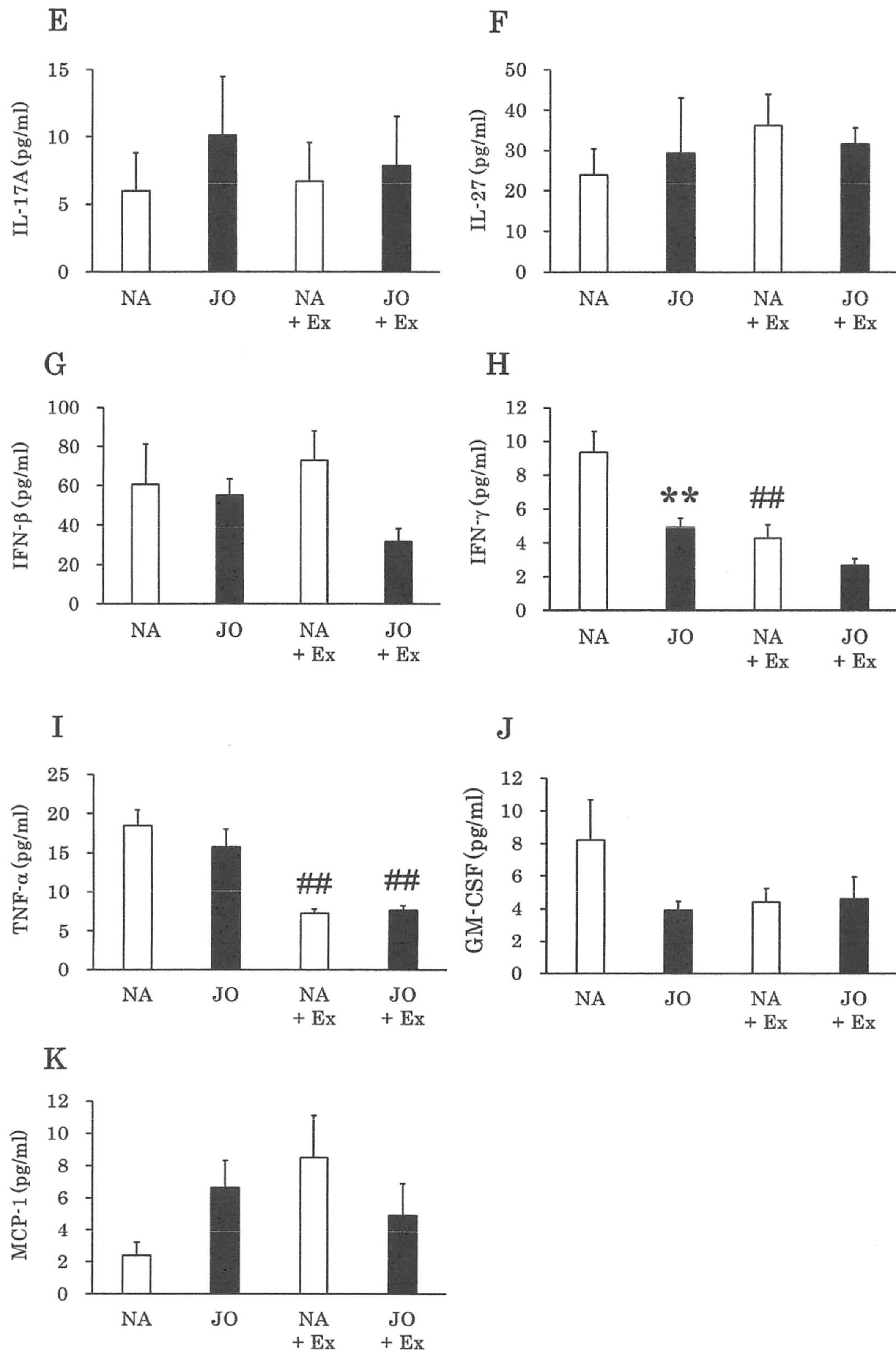


Figure 8. Plasma cytokine levels of (A) IL-1α, (B) IL-1β, (C) IL-6, (D) IL-10, (E) IL-17A, (F) IL-27, (G) IFN-β, (H) IFN-γ, (I) TNF-α, (J) GM-CSF, and (K) MCP-1 were measured by flow cytometry. NA, the

naïve control group; JO, the sedentary group with jojoba oil applied; NA + Ex, the exercise group with no oil applied; JO + Ex, the exercise group with jojoba oil applied; IL, interleukin; IFN, interferon; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1. Values are means ± SE, n = 7. $^{**}p < 0.01$ as compared to the NA group. $^{\#}p < 0.05$ as compared to the corresponding sedentary group. $^{\#\#}p < 0.01$ as compared to the corresponding sedentary group.

3.3.8. Correlation Between Plasma CK Levels and Time-to-Exhaustion With or Without Topical Application of Jojoba Oil

To investigate whether prolonged exercise duration is related to muscle damage, the correlation between plasma CK levels as a muscle damage marker and running time was confirmed (Figure 9). As a result, plasma CK levels were significantly correlated with the run time-to-exhaustion ($r = 0.723$, $p < 0.001$).

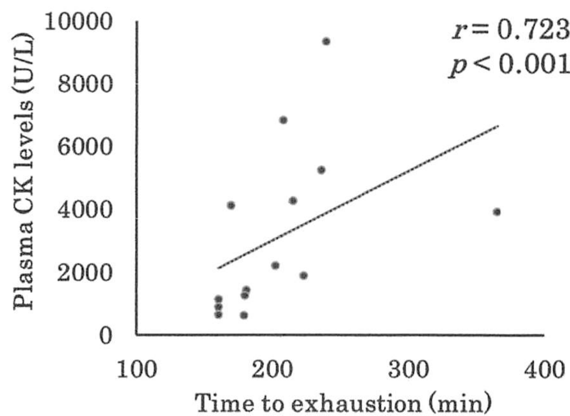


Figure 9. Correlation between plasma creatine kinase (CK) levels and the run time-to-exhaustion with and without topical application of jojoba oil. Plasma CK levels were significantly correlated with time-to-exhaustion ($r = 0.723$, $p < 0.001$). Correlations were determined using the Spearman's correlation analysis.

3.3.9. Correlation Between Plasma CK Levels and Inflammation-Related Genes Expressed in Skeletal Muscles With or Without Topical Application of Jojoba Oil

Since there was a significant correlation between the treadmill running time and plasma CK levels (Figure 9), the correlation between plasma CK levels and the expression levels of pro-inflammatory cytokines/enzymes (*Il-1b*, *Il-6*, and *iNOS*), and anti-inflammatory cytokines (*Il-1ra* and *Il-10*) was further analyzed.

As shown in Figure 10, plasma CK levels were significantly correlated with the expression levels of *Il-1b* ($r = 0.617$, $p < 0.01$), *Il-6* ($r = 0.874$, $p < 0.001$), *Il-10* ($r = 0.650$, $p < 0.001$), and *iNOS* ($r = -0.720$, $p < 0.001$) in the soleus muscle. Plasma CK levels were also significantly correlated with the expression levels of *Il-1b* ($r = 0.764$, $p < 0.001$), *Il-10* ($r = 0487$, $p < 0.01$), and *iNOS* ($r = -0.660$, $p < 0.001$) in the gastrocnemius muscle.

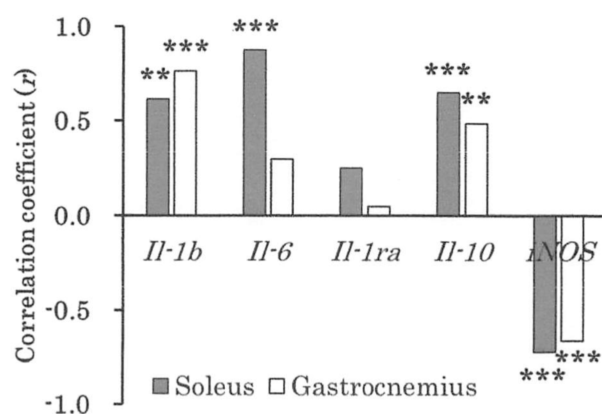


Figure 10. Correlation between plasma creatine kinase (CK) levels and inflammation-related genes expressed in soleus (slow-twitch muscle) and gastrocnemius muscles (fast-twitch muscle) with or without topical application of jojoba oil. Plasma CK levels were significantly correlated with the expression levels of *Il-1b*, *Il-6*, *Il-10*, and *iNOS* genes in the soleus muscle and with the expression levels of *Il-1b*, *Il-10*, and *iNOS* genes in the gastrocnemius muscle. Correlations were determined using Spearman's correlation analysis. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. Discussion

3.4.1. Effect of Topical Application of Undiluted Jojoba Oil Before Exercise on Endurance Performance and Biochemical Analysis of Plasma

The purpose of this study was to investigate whether the topical application of jojoba oil before running on a treadmill prevents exercise-induced muscle damage and affects the running time-to-exhaustion. Recently, it was reported that plasma NEFA levels were elevated 30 min after the transdermal administration of jojoba oil in mice (98). Based on the previous research, this study investigated whether jojoba oil applied 30 min before exercise prolongs the time-to-exhaustion during treadmill exercise. As a result, the data indicate that this did not affect the run time-to-exhaustion (Figure 2). The reason for this result can be inferred from the plasma biochemistry data (Figure 3). First, in the exercise groups (NA + Ex, JO + Ex), GLU and LA were lower than those in the sedentary groups, indicating that they were consumed as energy substrates. Moreover, there was no significant difference in plasma GLU and LA concentrations between the NA + Ex and JO + Ex groups. Plasma levels of IL-6 were higher in both the NA + Ex group and JO + Ex group than in the rest, but there was no significant difference between the two groups. Although IL-6 is widely known as an inflammatory cytokine, it has been reported that the physiological concentration of IL-6 also has an anti-inflammatory effect (100) and a metabolic enhancing effect (101, 102). In this experiment, there was no significant difference in the run time-to-exhaustion between the NA + Ex and JO + Ex groups. Therefore, we speculate that there was no difference in the effect of IL-6 on energy metabolism between these two groups. From a different point of view, since the topical application of jojoba oil does not improve endurance exercise capacity, it can be used for sports massage without worrying about doping regulations.

Furthermore, CK, which is a marker of muscle injury (103), ALT, AST, and ALP, which are markers of liver injury (104, 105), and BUN and CRE, which are markers of kidney injury, increased in both the NA + Ex and JO + Ex groups. Therefore, it can be inferred that the treadmill exercises were properly performed. The fact that no significant difference was observed between the two exercise groups with regards to the aforementioned markers implies that even if jojoba oil is applied before treadmill running, this procedure does not alleviate reversible organ damage, caused by exercise, to the liver, kidneys, and skeletal muscles.

The data show that plasma levels of NEFA were significantly lower in the JO group than in the NA group (Figure 3). These data are inconsistent with those of a previous study (98), but the reason might be that the time from applying jojoba oil to collecting blood was different between these two studies (30 min in the previous study and approximately 3 h in the present study). In this study, blood was collected at an average of 3 h after oil application because the time was aligned with the sampling of the corresponding exercise group. Furthermore, the difference in the results between the present study and the previous study might have been caused by circadian rhythms in lipid metabolism (106-109).

3.4.2. Effects of Exhaustive Exercise With or Without Topical Application of Jojoba Oil on the Expression Levels of Inflammation-Related Genes in Various Tissues/Organs

The purpose of the present study was to confirm whether jojoba oil applied before exercise could alleviate the inflammation caused by exercise. To clarify this research question, the effects of topical application of jojoba oil on treadmill running in mice were evaluated by measuring the expression of inflammation-related mRNAs in the soleus

muscle, gastrocnemius muscle, heart (cardiac muscle), and liver. The gene expression levels of *Il-1* and *Il-6* as inflammatory cytokines and *Il-1ra* and *Il-10* as anti-inflammatory cytokines were evaluated.

IL-1ra reportedly inhibits IL-1β signal transduction (110). IL-10 is an anti-inflammatory cytokine, which inhibits the synthesis of inflammatory cytokines, such as TNF-α (111). Therefore, the expression levels of *Il-1ra* and *Il-10* as anti-inflammatory cytokines were analyzed. Since NO synthase is induced in the inflammatory region by inflammatory cytokines and causes cytotoxicity, the expression levels of *iNOS* was also evaluated. Inducible NOS is widely known as a pro-inflammatory enzyme because it produces 20–50 times higher amounts of the free radical (nitric oxide) than the constitutive isoforms of NOS (112).

This study revealed that inflammatory cytokines showed that the expression levels of *Il-1b* and *Il-6* genes in the soleus muscle and liver were significantly increased by exhaustive exercise. Similarly, in the gastrocnemius muscle, the expression level of *Il-1b* was significantly increased by exhaustive exercise. In contrast, the expression levels of *Il-1b* and *Il-6* in the heart were not significantly increased by exhaustive exercise. We also analyzed whether jojoba oil, which has an anti-inflammatory effect, suppresses the gene expression of these inflammatory cytokines induced by exhaustive exercise. As a result, it was confirmed that the expression levels of *Il-1b* and *Il-6* in the soleus muscle and the expression levels of *Il-1b* in the gastrocnemius muscle were significantly suppressed by jojoba oil after exhaustive exercise (Figure 4, Figure 5). These results suggest that jojoba oil can be applied topically before exercise to reduce inflammation in the skeletal muscles of the lower extremities. In contrast, exercise-induced liver *Il-b* and *Il-6* gene expression was not suppressed by the topical application of jojoba oil.

In all organs evaluated in this study, the expression levels of *iNOS* were significantly decreased by exhaustive exercise. The topical application of jojoba oil had no effect on the expression levels of *iNOS* between the NA + Ex and JO + Ex groups. Notably, contrasting results from previous studies show that the expression level of *iNOS* is significantly increased by five consecutive days of treadmill running (113) but significantly reduced in the gastrocnemius muscle 2 h post-swimming in mice fed a high-fat diet (114). Therefore, these results imply that the expression level of *iNOS* decreases with a single exercise session but increases when forced exercise is performed every day without recovery of the damaged muscles.

Furthermore, as a mechanism of the anti-inflammatory action of jojoba oil, it was assumed that the expression level of anti-inflammatory cytokines might increase simultaneously with a decrease in the expression levels of inflammatory cytokines. Therefore, the expression levels of *Il-1ra* and *Il-10* were examined as anti-inflammatory cytokines. As a result, the expression level of *Il-10* in the soleus muscle and the liver was significantly increased by exhaustive exercise. In contrast, the expression level of *Il-1ra* was significantly increased only in the liver after exhaustive exercise. However, comparing the NA + Ex group and the JO + Ex group in the liver, the topical administration of jojoba oil did not enhance post-exercise anti-inflammatory cytokine gene expression in the liver. On the contrary, the expression levels of *Il-10* in the gastrocnemius muscle showed a significant increase only in the JO + Ex group after exercise, and thus, it might be possible that the topical application of jojoba oil increased the expression levels of *Il-10* in this muscle. The expression levels of soleus *Il-10* were significantly suppressed in the JO + Ex group as compared to those in the NA + Ex group. The reason for this phenomenon may be related to the gene expression of *Il-10* is linked

to increased expression of soleus *Il-6* (100).

In summary, we suggest that the mechanism by which jojoba oil exerts an anti-inflammatory effect on exercise-induced inflammation is via the suppression of inflammatory cytokines. This is because in both the sedentary and exercise groups, the topical application of jojoba oil did not significantly increase the expression of anti-inflammatory cytokines.

3.4.3. Quantitative Analysis of Inflammation-Related Plasma Cytokine Levels by Flow Cytometry

Summarizing the results of the flow cytometric analysis, IL-6 was the only inflammatory cytokine that was elevated by exercise among the cytokines measured in this study, and a decrease in its concentration in the blood due to the topical application of jojoba oil was not observed. Conversely, since IL-6 is a myokine secreted by muscle contraction (115), the soleus muscle might have contributed to the increase in the plasma levels of IL-6 during exhaustive exercise. Additionally, since cytokines are produced by various tissues and immune cells (116), other tissues could have contributed to the increase in plasma levels of IL-6. For example, shear stress increases because of the promotion of blood flow to skeletal muscle during exercise (117), and shear stress on the bovine endothelial cell monolayer induces IL-1β and IL-6 secretion (118).

The plasma concentration of IL-1, an inflammatory cytokine, was not increased by exercise, but exercise increased the expression levels of *Il-1* in both the soleus muscle and gastrocnemius muscle. Jojoba oil significantly suppressed the gene expression of these exercise-induced inflammatory cytokines. These results suggest that jojoba oil might suppress exercise-induced local inflammation in skeletal muscles. In addition,

previous studies have reported that exercise increases plasma IL-6 levels (119), and the results of the present study are consistent with those of previous studies. Plasma levels of inflammatory cytokines also increase in endotoxemia, such as sepsis, but it has been reported that TNF-α and IL-1β do not increase with the exercise-induced production of cytokines (120), and the results in this study were similar to those in previous reports. The increased plasma levels of IL-6 due to exercise not only reflect exercise-induced inflammation, but also indicate the involvement of intracellular glucose uptake in the promotion of lipid metabolism (121-123). Therefore, among the inflammatory cytokines measured in this study, the plasma levels of IL-6 might be particularly elevated after exercise.

In the resting group, the plasma levels of IFN-γ decreased in the JO group compared with those in the NA group. Further research is needed to elucidate the mechanisms responsible for this observation. IFN-γ is a cytokine secreted mainly from T cells and natural killer cells and acts by enhancing inflammation, and this observation is suggestive of the anti-inflammatory effect of jojoba oil. Since it is known that the immune system is temporarily suppressed by exhaustive exercise (124-126), the reason why the plasma levels of IFN-γ decreased in the NA + Ex group as compared to those in the NA group is because of the decrease in lymphocyte function due to exhaustive exercise. As a mechanism, it has been demonstrated that synthetic glucocorticoid inhibits IFN production (127-129). In addition, the increase in plasma levels of cortisol and epinephrine due to exercise might be associated with the inhibitory effect of type 1 helper T-cell cytokine (IFN-γ) production. Moreover, as mechanisms by which plasma levels of TNF-α decreased in the exercise groups, this could be due to exercise-induced release of epinephrine (130, 131) and IL-6 (119, 132) which can suppress the production

of TNF-α.

3.4.4. Correlation Between Plasma CK Levels and Inflammation-Related Genes Expressed in Skeletal Muscles With or Without Topical Application of Jojoba Oil

Exhaustive exercise causes muscle damage. The biochemical parameters measured in this study included CK, AST, and ALT, which are abundant in skeletal muscles and are useful as muscle damage markers. The plasma concentrations of these parameters increased after exercise (Figure 3). Plasma CK levels have been widely used in many studies as an index of muscle damage (103). Plasma levels of AST and ALT are also markers of liver damage, but are not skeletal muscle-specific enzymes. Therefore, we analyzed the correlation between plasma CK level as a muscle damage marker and running time by Spearman's correlation analysis and confirmed that the plasma CK level can be used as a muscle damage marker in this experimental system (Figure 9). Next, we examined the correlation between muscle damage (increased plasma CK levels) and the gene expression levels of inflammation-related cytokines in skeletal muscles. As a result, a correlation was found between plasma CK levels and *Il-1b* and *Il-6* expression in the soleus muscle and *Il-1b* expression in the gastrocnemius muscle. These results were similar to the results of the investigation of organs and genes in which expression levels were suppressed by the application of jojoba oil. From these results, it can be hypothesized that jojoba oil suppressed the expression of genes that correlate with exercise-induced muscle damage.

The present study also showed a correlation between plasma CK levels and *Il-10* expression in both soleus and gastrocnemius muscles. This might be related to the induction of plasma IL-10 via elevated plasma IL-6 concentrations (100, 132, 133). In the

gastrocnemius muscle, *Il-1b* was the only gene that was correlated with plasma CK levels, whereas in the soleus muscle, both *Il-6* and *Il-1b* were significantly correlated with plasma CK levels. This might be because slow-twitch muscle contributes more than fast-twitch muscle to the endurance exercise performed in this study. A negative correlation was found between elevated blood CK levels and the expression levels of *iNOS* in skeletal muscle, which might reflect a significant decrease in the expression levels of *iNOS* in skeletal muscles after exercise.

3.4.5. New Potential Therapeutic Strategy for Preventing Exercise-Induced Muscle Damage Using Topical Application of Jojoba Oil

Therapeutic strategies for exercise-induced muscle damage include stretching, massage, nonsteroidal anti-inflammatory drugs (NSAIDs), and nutritional support (92, 93, 95). Although NSAIDs are widely used in the treatment of inflammatory diseases and pain in the clinical setting, there are a number of side effects, including gastrointestinal bleeding (134, 135). Jojoba oil has been used topically as a moisturizing agent for skincare and lubricants for sports massage, but it has not been used as a medicinal substance. Transdermal administration has several advantages such as avoiding the first-pass effect, which results in a significant reduction in the drug concentration via hepatic metabolic enzymes before the drug reaches the blood circulation (136-138). It is also absorbed slowly through the skin, which exerts a sustained effect (139), results in a stable and long-lasting effective blood concentration (137, 138), and is a simple and non-invasive administration method (137, 138). Transdermal absorbents, such as a scopolamine (hyoscine) patch for the treatment of motion sickness, nitroglycerin for angina pectoris, transdermal clonidine for the

treatment of hypertension, transdermal estradiol for female hormone-replacement therapy, transdermal fentanyl for the treatment of pain, nicotine patches for smoking cessation, and transdermal testosterone for hypogonadism, are widely used as medical treatments (136, 140).

Jojoba oil is very similar to the wax ester that accounts for approximately 25% of human sebum (64). Therefore, it fits well on the skin and has been proven safe by the experience of many people who have used it. Furthermore, since it has been reported that massage alone has an anti-inflammatory effect (141), it is possible that sports massage using jojoba oil could have a synergistic anti-inflammatory effect compared to that with either jojoba oil or massage. In the future, it is necessary to clarify this synergistic effect by verifying this application based on research on sports massage in humans.

3.5. Conclusions

This study indicates that topical application of jojoba oil 30 min before treadmill running did not affect the endurance capacity of mice. With regard to exhaustive exercise-induced inflammation, there were no significant changes in plasma cytokine levels due to the topical application of jojoba oil among the exercise groups. However, the topical application of jojoba oil 30 min before treadmill running significantly decreased the mRNA levels of some pro-inflammatory cytokines (*Il-1β* and *Il-6*) in skeletal muscles, and especially in the slow-twitch muscle, which contributes to endurance exercise. Taken together, the topical application of jojoba oil 30 min before exhaustive exercise might reduce exercise-induced inflammation. These results suggest that sports massage using jojoba oil may be useful for preventing exercise-induced muscle damage and

inflammation.

Chapter 4.

Effects of Topical Application of Wintergreen Oil on Exercise-Induced Inflammation in Mice

4.1. Introduction

Exhaustive exercise triggers the production of pro-inflammatory cytokines, which in turn increases oxidative stress in active muscles, resulting in a vicious cycle that further promotes inflammation (10). Therefore, alleviating the symptoms of muscle damage and inflammation may be useful for athletes who need to recover as quickly as possible for subsequent games (93, 94). Recently, sports massage was reported to alleviate exercise-induced inflammation (24). In sports massage, essential oils diluted with carrier oil or undiluted carrier oils are used as massage oils. Carrier oils are vegetable oils that dilute the essential oils used in aromatherapy, such as jojoba, sweet almond, and macadamia nut oils, among others. Of these, jojoba oil is a wax ester, whose composition is similar to that of human sebum. Indeed, approximately 25% of human sebum is composed of wax esters (64). Jojoba oil is also resistant to oxidation compared with other carrier oils and can be used for a long time after opening the package. This makes it easy for athletes to purchase for self-care. For this reason, jojoba oil is widely used in sports aromatherapy massage.

In this study, we defined sports aromatherapy massage as a method of performing sports massage using an essential oil diluted with a carrier oil. With sports aromatherapy massage, it is expected that the essential oils will exert biological activity

in addition to the effects of mechanical stimulation. Essential oils are natural volatile oils extracted from various parts of the plant and contain natural compounds in high concentrations and have various aromatic components. Some of these natural compounds have anti-inflammatory activity, and they impart this characteristic to some essential oils such as wintergreen, tea tree, lavender, mint, and ginger oils (142-144). Among these essential oils, wintergreen oil (also known as oil of wintergreen), which contains a high concentration (more than 99%) of methyl salicylate, is widely known among therapists as an essential oil with anti-inflammatory properties. After percutaneous absorption, methyl salicylate is metabolized to salicylic acid, which exerts anti-inflammatory effects in the body (145, 146). Methyl salicylate has been used as an active ingredient in many pharmaceutical and commercial brands of ointments, lotions, liniments, and medicated oils for topical application to relieve musculoskeletal pain (147). The concentration of methyl salicylate in commercial pain relief patches is 1–20%. In general aromatherapy for relaxation purposes, essential oils are diluted to about 1% with carrier oil. In contrast, in sports aromatherapy massage, essential oils, are diluted to a high concentration of approximately 5–10% to achieve the effects of their active ingredients. Further, a somewhat special application is the use of a few drops of undiluted wintergreen or clove oil on the affected area to relieve pain. Therefore, in this study, we used undiluted wintergreen oil and that diluted to 10% with jojoba oil. Currently, few studies on sports aromatherapy massage have been reported, and the concentration of essential oils in massage oil is determined based on experience. Furthermore, whether essential oils with anti-inflammatory properties have an inhibitory or mitigating effect on exercise-induced inflammation remains unknown. Therefore, the present study aimed to investigate whether the topical application of

wintergreen oil before treadmill running could alleviate exercise-induced inflammation and determine if this would affect the run time-to-exhaustion. In addition, wintergreen oil can be used in undiluted solutions, and thus, the safety of wintergreen oil transdermal absorption was evaluated based on blood biochemical data.

4.2 Materials and Methods

4.2.1 Ethics Statement

All animals were cared for in accordance with Law No. 105 and Notification No. 6 of the Japanese Government, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Waseda University (2019-A132).

4.2.2 Animals

Seven-week-old male hairless mice (Hos-HR-1) were purchased from Hoshino Laboratory Animals, Inc. (Bando, Japan) through Japan SLC, Inc. (Hamamatsu, Japan) and housed in a breeding room for at least 1 week. All mice were 8 weeks of age (28.2–35.4 g; average = 31.6 g) at the beginning of the experiments. All mice were housed under specific pathogen-free conditions with a standard commercial diet (MF, Oriental yeast, Tokyo, Japan), and water was provided *ad libitum*. The mice were housed at 22–24°C with 50–60% relative humidity, under a light-dark cycle (lights on at 08:00 and off at 20:00).

4.2.3. Chemicals

We used jojoba oil, wintergreen oil diluted with jojoba oil to 10% (v/v), and undiluted wintergreen oil. Jojoba oil was obtained from Kenso-Igakusha (Yamanashi, Japan; Lot.

GL15A). It is a fatty acid-containing wax ester, is different from common vegetable oils, and is rich in triglycerides (TGs). Based on the manufacturer's package insert, the ingredients of the jojoba oil used in this study are listed in Table 9. Wintergreen oil was purchased from Kenso-Igakusha (Yamanashi, Japan; Lot. GPL132). Based on the manufacturer's package insert, the ingredients of the wintergreen oil used in this study are listed in Table 10. The structural formula of methyl salicylate, which is the main component of wintergreen oil, is shown in Figure 11.

Table 9. Fatty acid composition of jojoba oil.

Name	MW [g/mol]	Content [%]
Eicosenoic acid	310.51	73.4
Erucic acid	338.57	14.7
Oleic acid	282.47	8.3

MW, molecular weight.

Table 10. Components of wintergreen oil.

Name	MW [g/mol]	Content [%]
Methyl salicylate	152.15	99.32
Linalool	154.25	0.30
Ethyl salicylate	166.17	0.04
Eugenol	164.20	0.03

MW, molecular weight.

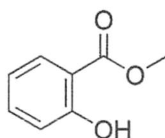


Figure 11. Structural formula of methyl salicylate.

4.2.4. Experimental Protocol

One week before exhaustive exercise, all exercise-group mice were acclimated to running on a motorized treadmill at 0% grade and a speed of 15 m/min for 10 min. On the day of the experiment, all mice were randomly assigned to one of the four groups as follows: the naïve control group (NA, n = 8), the sedentary group with 10% wintergreen oil (10%WG, n = 8), sedentary group with undiluted wintergreen oil applied (WG, n = 8), the exercise group with no oil applied (NA + Ex, n = 8), the exercise group with 10% wintergreen oil applied (10%WG + Ex, n = 8), and the exercise group with undiluted wintergreen oil applied (WG + Ex, n = 6). Mice were topically administered 4 μL of 10% wintergreen oil or undiluted wintergreen oil per gram of body weight to the dorsal skin, 30 min before treadmill running. In this protocol, these oils were administered 30 min before the start of running, based on previous studies (67, 68, 98, 148).

The endurance exercise was performed on a motorized treadmill (Natsume Seisakusyo Co., Ltd., Tokyo, Japan), and the run time-to-exhaustion was recorded. Mice in the NA + Ex, 10%WG + Ex, and WG + Ex groups were subjected to treadmill running at 10 m/min for 15 min, followed by 15 m/min and 20 m/min for 15 min each and then 24 m/min at a constant 7% grade until exhaustion. During exercise, the sedentary group was subjected to fasting to adjust for feeding conditions. Exhaustion was defined as the inability to continue regular treadmill running despite stimulation through repeated gentle tapping with a silicon rubber spatula on the back of the mouse. The endpoint of exhaustion was determined by a treatment-blinded single observer for all run time-to-exhaustion tests within the study to minimize observer bias. In addition, electric shock was not administered to avoid stressing the mice. This method is a humane improvement and reduces the confounding effect of negative reinforcement on blood cytokine levels

caused by the use of shock grids (99). Immediately after exhaustion, blood samples from the heart were collected using heparin under light anesthesia with inhalant isoflurane (Abbott, Tokyo, Japan), and isolated muscle tissues were immediately frozen in liquid nitrogen. Plasma was obtained from blood samples by centrifugation at 1,500 × *g* for 15 min at 4°C and the supernatant was separated. These samples were stored at −80°C until analysis.

4.2.5. Biochemical Analysis of Plasma

Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), creatine kinase (CK), TG, non-esterified fatty acids (NEFA), glucose (GLU), and lactic acid (LA) were measured at Kotobiken Medical Laboratories (Tsukuba, Japan). The plasma levels of ALT and AST were determined by the JSCC transferable method using L-Type ALT J2 and L-Type AST J2, respectively (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd., Tokyo, Japan). The plasma ALP level was measured by the IFCC transferable method using L-Type ALP IFCC (FUJIFILM Wako Pure Chemical Corporation) with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.). The plasma level of BUN was determined based on the urease-GlDH method using L-Type UN·V (FUJIFILM Wako Pure Chemical Corporation) with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.). The plasma CRE level was determined according to an enzymatic method using Determiner L CRE (Hitachi Chemical Diagnostics Systems Co., Ltd., Tokyo, Japan) with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.). The plasma level of CK was analyzed using the JSCC transferable

method using L-Type CK (FUJIFILM Wako Pure Chemical Corporation) with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.). The plasma levels of TG and NEFA were determined based on an enzymatic method using Determiner C-TG (Hitachi Chemical Diagnostics Systems Co.) and NEFA-HR II (FUJIFILM Wako Pure Chemical Corporation), respectively, with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.). The plasma GLU level was measured by the hexokinase-G-6-PDH method using L-Type Glu 2 (FUJIFILM Wako Pure Chemical Corporation) with a BioMajesty™ automated analyzer (JCA-BM9130; JEOL Ltd.). The plasma level of LA was determined according to an enzymatic method using Determiner LA (Hitachi Chemical Diagnostics Systems Co.) with a BioMajesty™ automated analyzer (JCA-BM8060; JEOL Ltd.).

4.2.6. RNA Isolation and Gene Expression Analysis Using Real-Time Polymerase Chain Reaction (Real-Time PCR)

Immediately after the tissue samples were harvested, each sample was frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed. Total RNA was extracted from the soleus muscle (slow-twitch muscle), gastrocnemius muscle (fast-twitch muscle), heart, and liver using acid guanidinium thiocyanate-phenol-chloroform extraction (149, 150) combined with the bead homogenization method using a Shake Maser Neo (Bio Medical Science, Tokyo, Japan). The purity and quality of the total RNA from each sample were assessed based on the 260:280 nm optical density ratio, using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA). Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA), in accordance with the

manufacturer's instructions. PCR was carried out with the StepOnePlus™ system (Applied Biosystems), using the Fast SYBR® Green Master Mix (Applied Biosystems). The cycling conditions were as follows: 10 s at 95°C for denaturation, followed by 45 cycles at 95°C for 5 s, 57°C for 10 s (annealing), and finally, 72°C for 10 s. Data were normalized to *β-actin* levels as an internal standard, using the calibration curve method. The genes and primers used are listed in Table 11.

Table 11. Primers used for real-time polymerase chain reaction analysis.

Gene	Accession no.	Forward	Reverse
Il-1b	NM_008361.4	TGCCACCTTTTGACAGTGATG	TGTGCTGCTGCGAGATTTGA
Il-1ra	NM_031167.5	TGTGCCAAGTCTGGAGATGA	TTCTTTGTTCTTGCTCAGATCAGT
Il-6	NM_001314054.1	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
Il-10	NM_010548.2	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
iNOS	NM_001313922.1	GGGCTGTCACGGAGATCA	CCATGATGGTCACATTCTGC
Cpt-1a	XM_006531658.3	CCAGGCTACAGTGGGACATT	AAGGAATGCAGGTCCACATC
Actb	NM_007393.5	CCTCCCTGGAGAAGAGCTATG	TTACGGATGTCAACGTCACAC

Il-1b, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase; *Cpt-1a*, carnitine palmitoyl transferase-1α; *Actb*, β-actin.

4.2.7. Quantitative Analysis of Inflammation-Related Plasma Cytokine Levels

Plasma cytokine levels were determined using the LEGENDplex™ Mouse Inflammation Panel (Cat No. 740150; BioLegend, San Diego, CA, USA) in accordance with the manufacturer's instructions. Specifically, the plasma levels of monocyte chemoattractant protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-β, IFN-γ, interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, and tumor necrosis factor (TNF)-α were measured. The analysis was performed with a BD LSRFortessa Special Order Research Product analytical

cytometer (Becton Dickinson, San Jose, CA, USA), and data were evaluated with LEGENDplex™ Data Analysis software.

4.2.8. Statistical Analysis

Values are presented as the mean ± standard error. For statistical analysis, differences in means among groups were analyzed by a one-way analysis of variance, and Tukey's *post-hoc* test was performed to determine the significance among the means. Statistical analysis was performed using the SPSS 24.0 software package (IBM Japan Inc., Tokyo, Japan). Statistical significance was defined as $p < 0.05$.

4.3. Results

4.3.1. Effects of Topical Application of Undiluted Wintergreen Oil and 10% Wintergreen Oil Before Exercise on Endurance Performance

As shown in Figure 12, the WG + Ex group with topical application of undiluted wintergreen oil showed a significantly shorter run time than the NA + Ex and 10% WG + Ex groups. Additionally, topical application of 10% wintergreen oil (10%WG + Ex) also significantly reduced the run time compared with the NA + Ex group. The run times-to-exhaustion in the NA + Ex, 10%WG + Ex, and WG + Ex groups were 225 ± 23 min, 160 ± 12 min, and 54 ± 2.7 min, respectively.

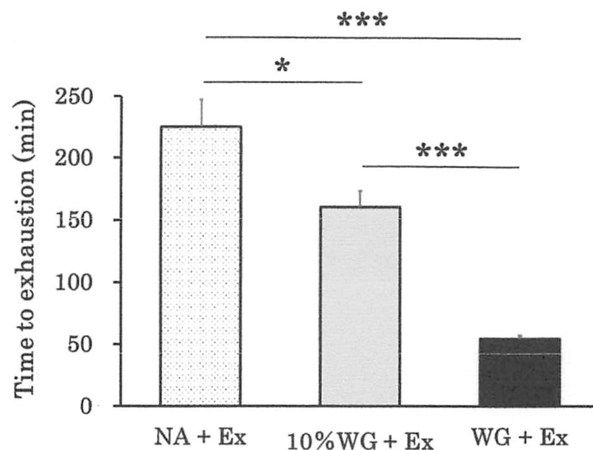


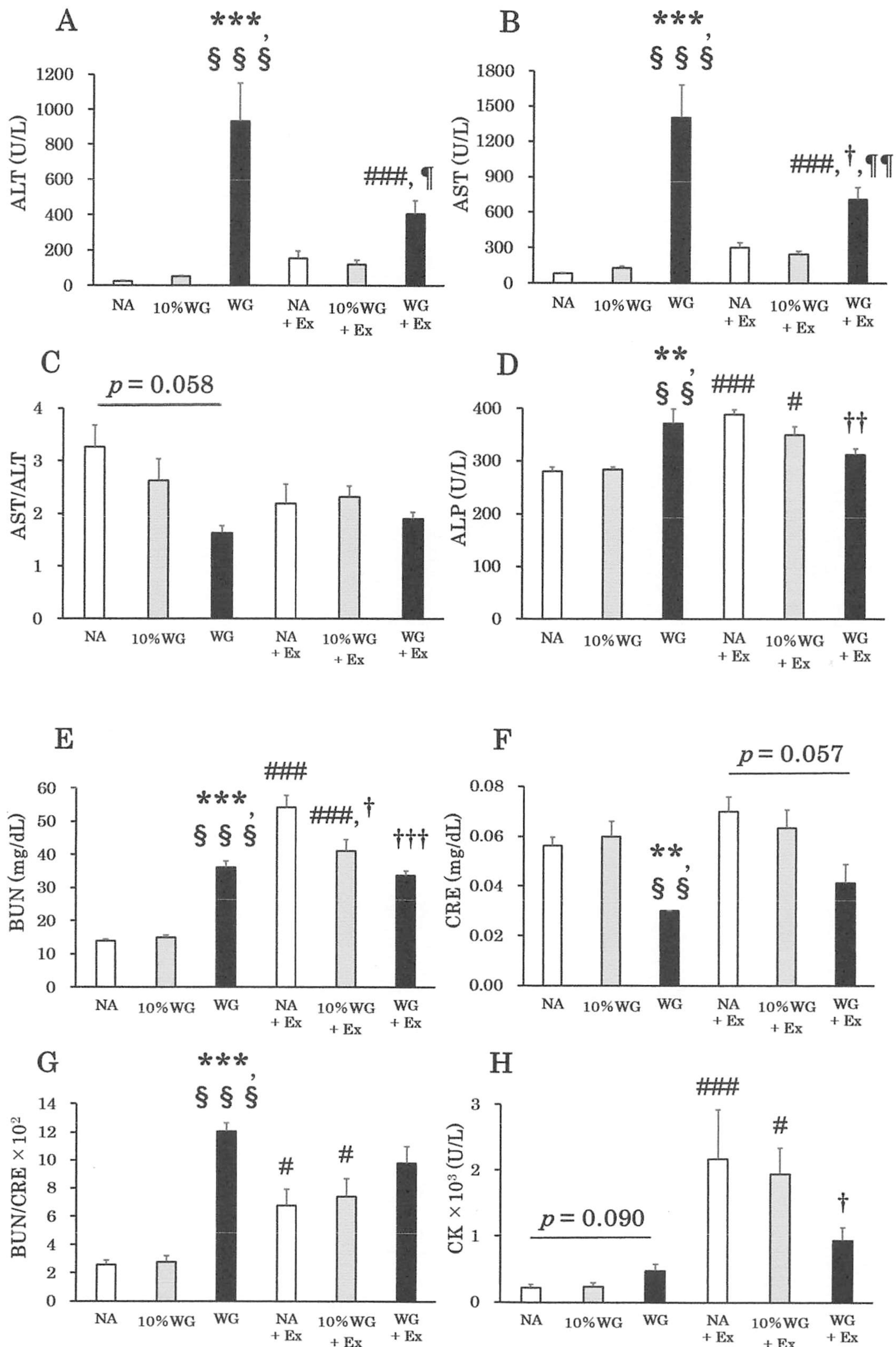
Figure 12. Run time-to-exhaustion in the exercise group with no oil applied (NA + Ex, n = 7), the exercise group with 10% wintergreen oil applied (10%WG + Ex, n = 9), and the exercise group with undiluted wintergreen oil applied (WG + Ex, n = 8). In the 10% WG+Ex group, mice were treated with wintergreen oil diluted to 10% (v/v) in jojoba oil. Values are presented as the mean ± standard error. N.S., not significant. $^{**}p < 0.01$. $^{***}p < 0.001$.

4.3.2. Biochemical Analysis of Plasma

Figures 13 A–L show the plasma biochemistry data among the sedentary and exercise groups in the absence or presence of two concentrations of wintergreen oil applications. We measured the plasma levels of ALT, AST, and ALP as markers of acute liver injury (Figures 13 A–D). As a result, all three were significantly increased in the WG group compared with the levels in the NA and 10%WG groups. In the exercise groups, the plasma levels of ALT and AST in the WG + Ex group were significantly increased compared with those in the NA + Ex and 10%WG + Ex groups. Furthermore, the plasma levels of AST in the WG + Ex group were significantly decreased compared with those in the corresponding sedentary group. In contrast, the plasma levels of ALP were significantly higher in the NA + Ex and 10%WG + Ex groups than in the corresponding sedentary groups. In addition, the plasma levels of ALP in the WG + Ex group were significantly decreased compared with those in the NA + Ex group.

We also measured the plasma levels of BUN and CRE as markers of kidney injury (Figures 13 E–G). The plasma levels of BUN and BUN/CRE in the WG group were significantly higher than those in the NA and 10%WG groups, whereas the plasma levels of CRE in the WG group were significantly lower than those in the NA and 10%WG groups. The plasma BUN levels and BUN/CRE were significantly higher in the NA + Ex and 10%WG + Ex groups than in the corresponding sedentary groups. Further, the plasma BUN levels were significantly lower in the 10%WG + Ex and WG + Ex groups than in the NA + Ex group. The plasma CK levels tended to increase in the WG sedentary group ($p = 0.090$). In contrast, the plasma levels of CK were significantly increased in the NA + Ex and 10%WG + Ex groups as compared with those in the corresponding sedentary groups.

Figures 13 I–L show biochemical data related to metabolism. Plasma NEFA levels were significantly higher in the 10%WG + Ex group than in the NA + Ex group and significantly lower in the WG + Ex group than in the 10%WG + Ex group. In the sedentary groups, the plasma GLU levels were significantly lower in the WG group than in the NA group. In contrast, the plasma GLU levels were significantly lower in the NA + Ex group and the 10%WG + Ex group than in the corresponding sedentary groups. The plasma LA levels in sedentary groups were significantly higher in the WG group than in the NA group and the 10%WG group. In contrast, the plasma LA levels were significantly lower in the NA + Ex group and 10%WG + Ex than in the corresponding sedentary groups. However, the plasma LA levels in the WG + Ex group were significantly higher than those in the NA + Ex and 10%WG + Ex groups.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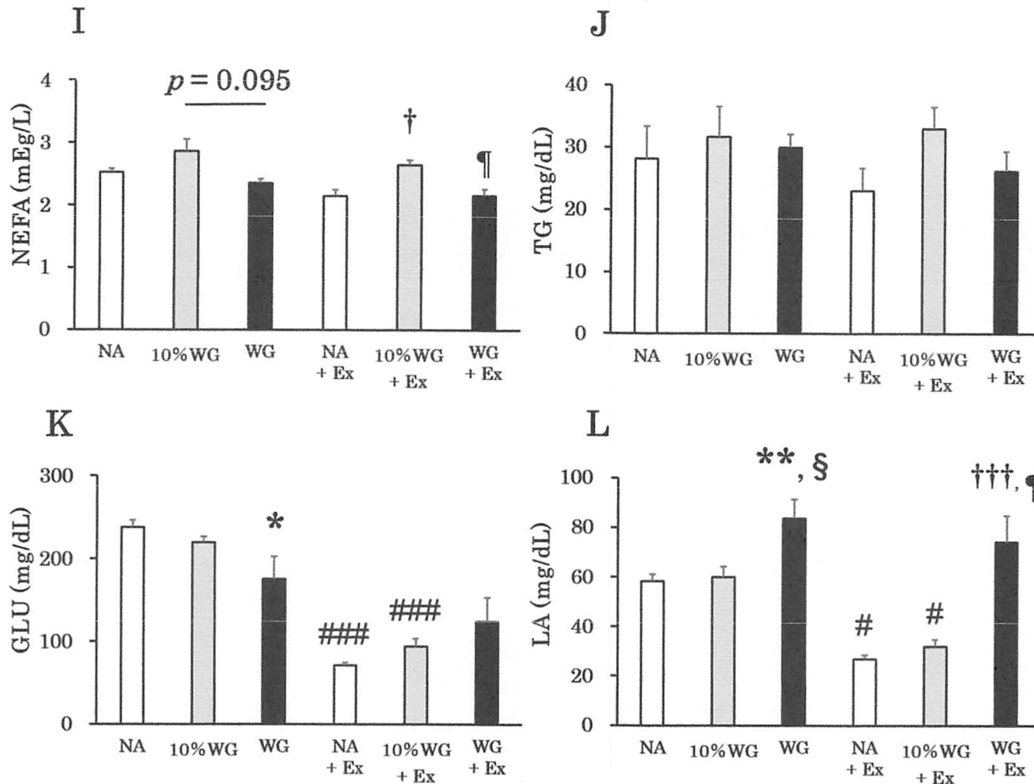


Figure 13. Plasma levels of (A) ALT, (B) AST, (C) AST/ALT, (D) ALP, (E) BUN, (F) CRE, (G) BUN/CRE, (H) CK, (I) NEFA, (J) TG, (K) GLU, and (L) LA. In the exercise groups, blood samples were obtained from the heart immediately after exhaustion. In the sedentary groups, blood samples were obtained at the same time as that in the exercise group for comparison. While the exercise groups were running, the sedentary groups were fasting to adjust for feeding conditions. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRE, creatinine; CK, creatine kinase; TG, triglyceride; NEFA, non-esterified fatty acids; GLU, glucose; LA, lactic acid. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{**}p < 0.01$ compared with the NA group. $^{***}p < 0.001$ compared with the NA group. $^{\#}p < 0.05$ compared with the corresponding sedentary group. $^{\#\#}p < 0.01$ compared with the corresponding sedentary group. $^{\#\#\#}p < 0.001$ compared with the corresponding sedentary group. $^{\dagger}p < 0.05$ compared with the NA + Ex group. $^{\dagger\dagger}p < 0.01$ compared with the NA + Ex group. $^{\dagger\dagger\dagger}p < 0.001$ compared with the NA + Ex group. $^{\S\S}p < 0.01$ compared with 10%WG group. $^{\S\S\S}p < 0.001$ compared with 10%WG group. $^{\P}p < 0.05$ compared with 10%WG + Ex group. $^{\P\P}p < 0.01$ compared with 10%WG + Ex group. $^{\P\P\P}p < 0.001$ compared with 10%WG + Ex group.

4.3.3. Effects of Exhaustive Exercise With or Without the Topical Application of Wintergreen Oil on the Expression Levels of Inflammation-Related Genes in the Soleus Muscle

In this study, we measured inflammation-related mRNA expression levels in various organs/tissues to assess the effect of the topical application of wintergreen oil on treadmill running in mice. In the soleus muscle (red, slow-twitch muscle), the expression levels of *Il-1b*, *Il-6*, and *Il-10* were significantly increased by exhaustive exercise (Figure 14). In contrast, the 10%WG + Ex and WG + Ex groups did not show a significant increase in the expression levels of these genes after treadmill running. The gene expression levels of *Il-1b*, *Il-6*, and *Il-10* were significantly lower in the 10%WG + Ex and WG + Ex groups than in the NA + Ex group. Further, the expression levels of *iNOS* in the 10%WG + Ex and WG + Ex groups were significantly higher than those in the NA + Ex group. However, there were no significant differences in *Il-1ra* expression levels among the sedentary and exercise groups. In addition, in the comparison of sedentary groups, no changes in the expression levels of inflammation-related genes did not change after wintergreen oil was applied.

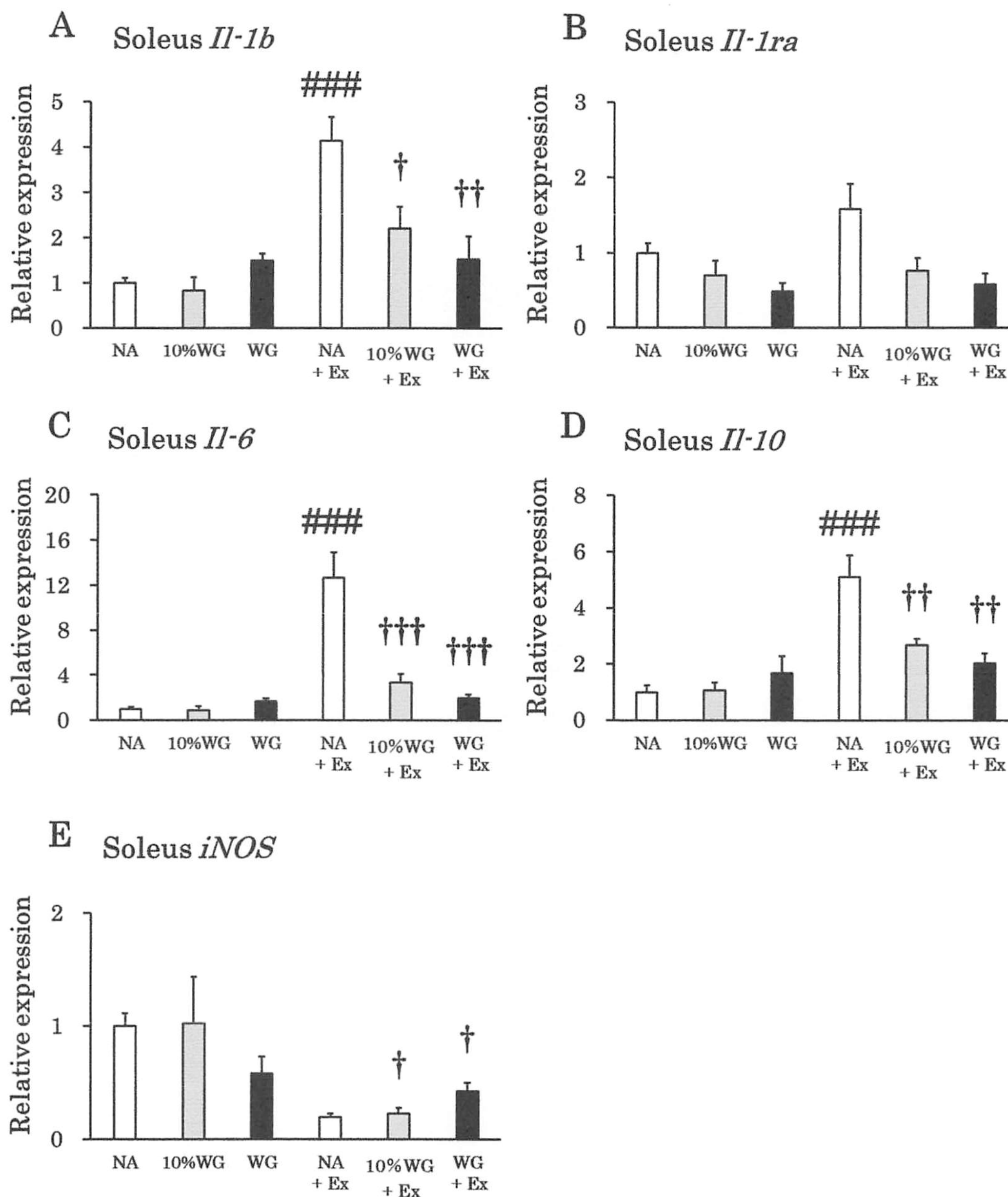


Figure 14. Gene expression levels of inflammation-related cytokines and *iNOS* in the soleus muscle. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied. *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{###}p < 0.001$ compared with the corresponding sedentary group. $^{†}p < 0.05$ compared with the NA + Ex group. $^{††}p < 0.01$ compared with the NA + Ex group. $^{†††}p < 0.001$ compared with the NA + Ex group.

4.3.4. Effects of Exhaustive Exercise With or Without the Topical Application of Wintergreen Oil on the Expression Levels of Inflammation-Related Genes in the Gastrocnemius Muscle

The expression levels of *Il-1b* in the gastrocnemius muscle (fast-twitch muscle) were significantly increased in the NA + Ex and the 10%WG + Ex groups compared with those in the corresponding sedentary groups. In contrast, the topical application of undiluted wintergreen oil resulted in higher *Il-1b* expression than that in the NA and the 10%WG groups. Further, the expression levels of *Il-6* in the WG group were significantly higher than those in the 10%WG + Ex group. However, there were no significant changes in *Il-6* expression levels among the exercise groups. Moreover, the expression level of *Il-10* in the gastrocnemius muscle was significantly decreased only in the WG + Ex group compared with the levels the NA + Ex group, and there was no significant difference in gene expression levels among the sedentary groups. The expression level of *iNOS* in the gastrocnemius muscle did not change significantly among the sedentary groups, but gene expression levels in the NA + Ex group decreased significantly. In contrast, the expression level of *iNOS* was significantly higher in the 10%WG + Ex and the WG + Ex groups than in the NA + Ex group. The differences in *Il-1ra* expression levels among the sedentary and exercise groups were not significant.

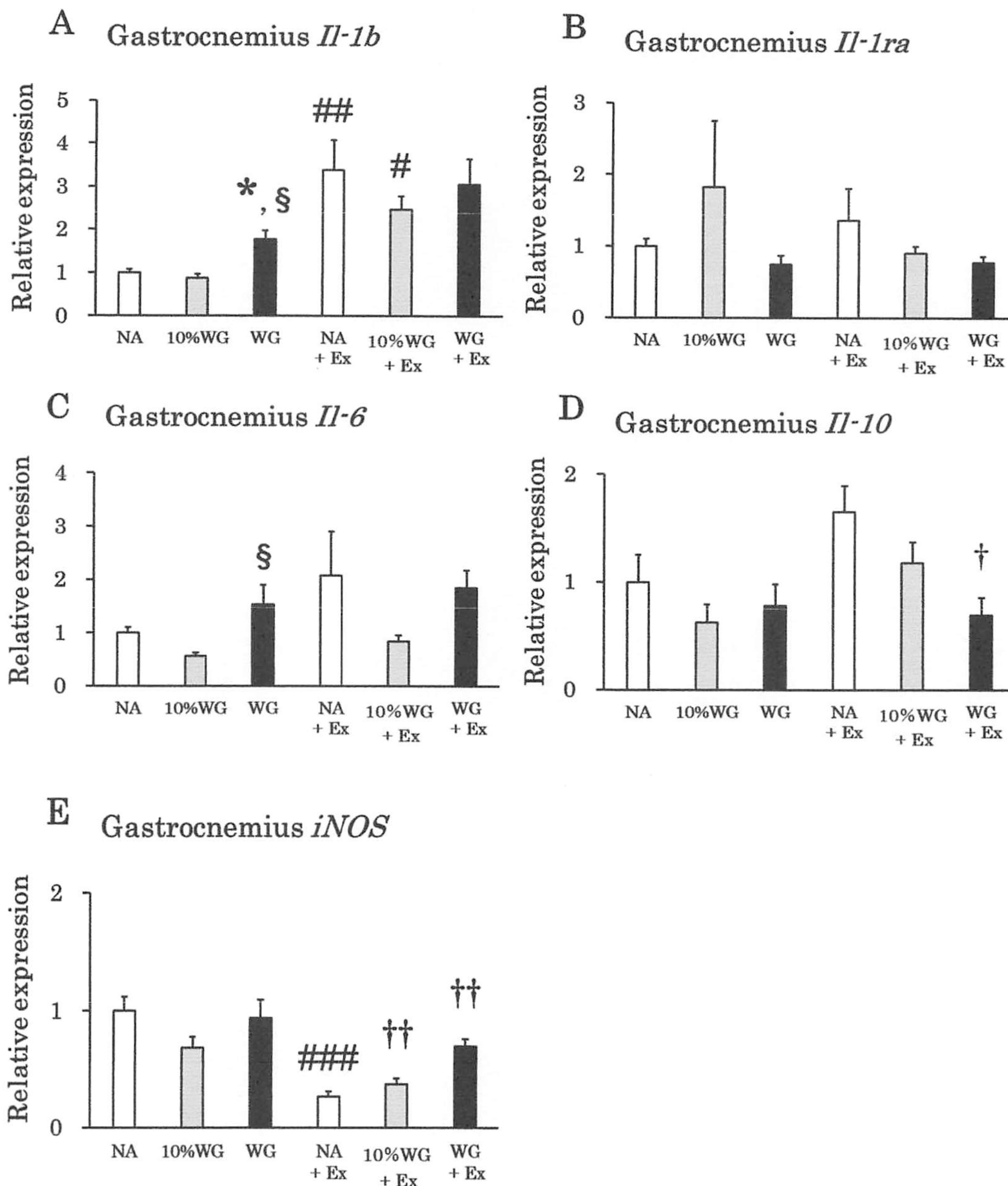


Figure 15. Gene expression levels of inflammation-related cytokines and *iNOS* in the gastrocnemius muscle. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied. *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{*}p < 0.05$ compared with the NA group. $^{\S}p < 0.05$ compared with the 10%WG group. $^{\#}p < 0.05$ compared with the corresponding sedentary group. $^{\#\#}p < 0.01$ compared with the corresponding sedentary group. $^{\#\#\#}p < 0.001$ compared with the corresponding sedentary group. $^{\dagger}p < 0.05$ compared with the NA + Ex group. $^{\dagger\dagger}p < 0.01$ compared with the NA + Ex group.

4.3.5. Effects of Exhaustive Exercise With or Without the Topical Application of Wintergreen Oil on the Expression Levels of Inflammation-Related Genes in the Heart

The expression levels of *Il-1b* in the WG group were significantly increased compared with those in the NA and 10%WG groups. Further, the expression levels of *Il-1b* in the WG + Ex group were significantly increased compared with those in the NA + Ex and 10%WG + Ex groups. The expression levels of *Il-6* in the WG group were significantly higher than those in the NA +Ex group. However, there were no significant changes in *Il-6* expression levels among the exercise groups.

The expression levels of *Il-10* in the WG group were significantly higher than those in the NA +Ex and 10%WG + Ex groups. The expression levels of *Il-10* in the NA + Ex and 10%WG + Ex groups were significantly increased compared with those in the corresponding sedentary groups. In contrast, in the exercise group, the expression levels of *Il-10* were significantly lower in the 10%WG + Ex and WG + Ex groups than in the NA + Ex group. The expression levels of *iNOS* in the heart were significantly decreased in the WG group compared with those in the NA group, and the expression levels of *iNOS* in the NA + Ex group were significantly lower than those in the NA group. There were no significant differences in *Il-1ra* expression levels among the sedentary and exercise groups.

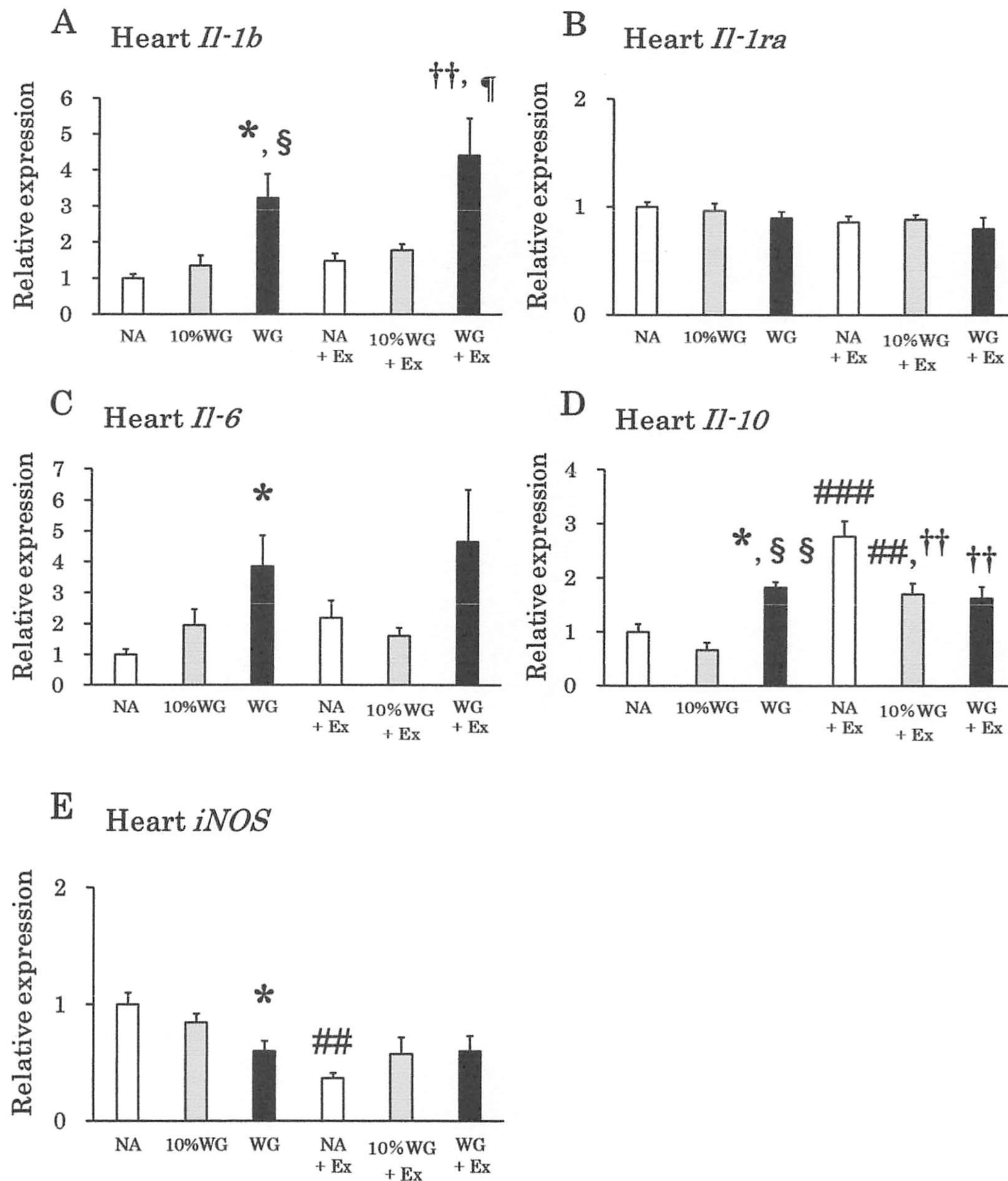


Figure 16. Gene expression levels of inflammation-related cytokines and *iNOS* in the heart. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied. *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{*}p < 0.05$ compared with the NA group. $^{§}p < 0.05$ compared with the 10%WG group. $^{§§}p <$

0.01 compared with the 10%WG group. ##$p < 0.01$ compared with the corresponding sedentary group. ###$p < 0.001$ compared with the corresponding sedentary group. ††$p < 0.01$ compared with the NA + Ex group. ¶$p < 0.05$ compared with the 10%WG + Ex group.

4.3.6. Effects of Exhaustive Exercise With or Without the Topical Application of Wintergreen Oil on the Expression Levels of Inflammation-Related Genes in the Liver

The expression levels of *Il-1ra* in the liver were significantly increased in the EX + NA group relative to those in the NA group. In addition, the expression levels of *Il-1ra* were lower in the 10%WG + Ex and WG + Ex groups than in the NA + Ex group. In the sedentary groups, the expression levels of *Il-1b*, *Il-6*, and *Il-10* were significantly increased in the WG group compared with those in the NA and 10%WG groups. In contrast, in the exercise groups, the expression levels of *Il-1b*, *Il-6*, and *Il-10* were significantly increased in the NA + Ex and 10%WG + Ex groups compared with those in the corresponding sedentary groups. In addition, the expression levels of *Il-10* in the WG + Ex group were significantly lower than those in the NA + Ex group. There were no significant differences in *Il-1ra* expression levels among the sedentary groups.

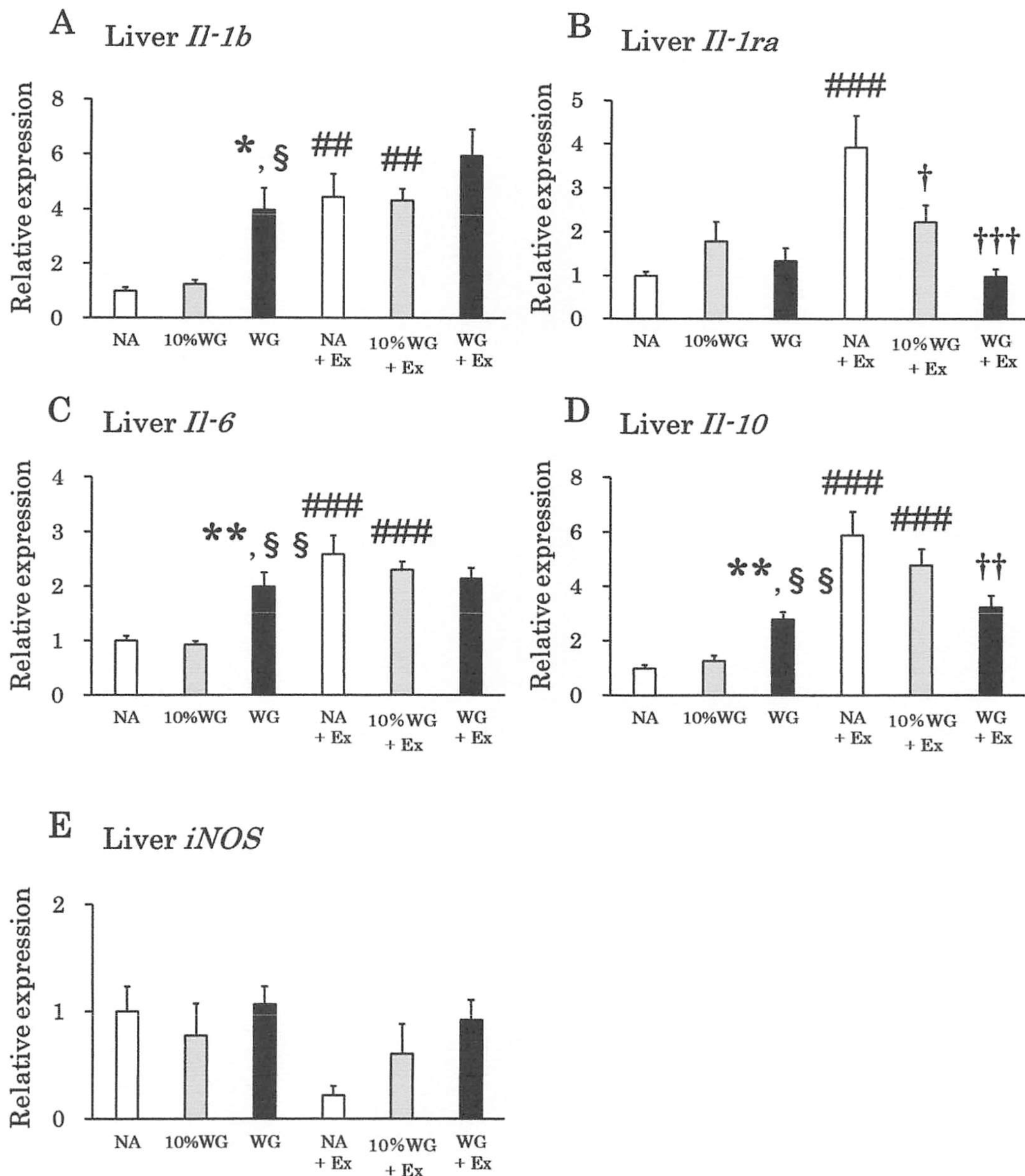
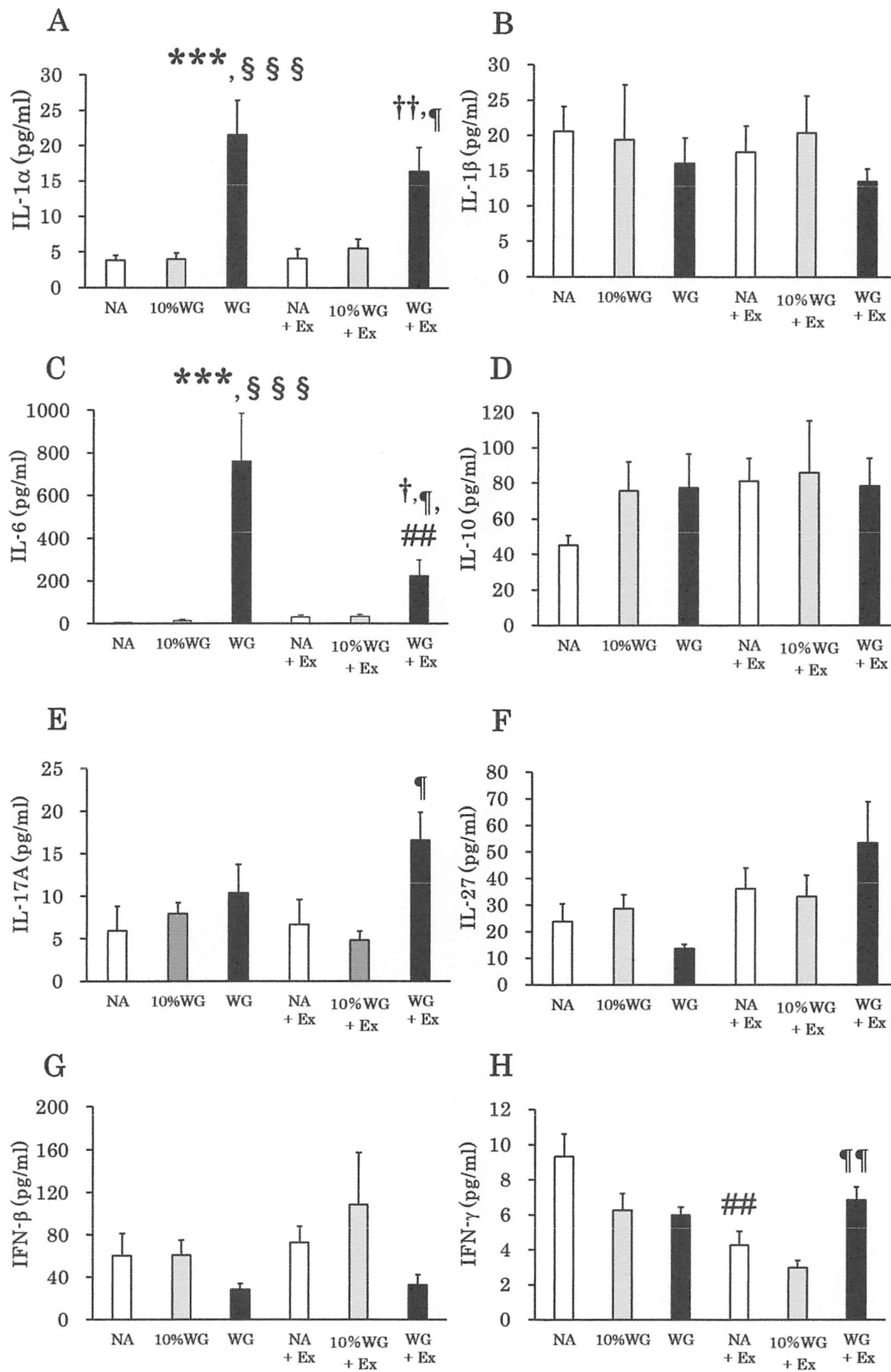


Figure 17. Gene expression levels of inflammation-related cytokines and *iNOS* in the liver. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied. *Il-1b*, interleukin 1β; *Il-1ra*, interleukin 1 receptor antagonist; *Il-6*, interleukin 6; *Il-10*, interleukin 10; *iNOS*, inducible nitric oxide synthase. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{*}p < 0.05$ compared with the NA group. $^{**}p < 0.01$ compared with the NA group. $^{\S}p < 0.05$ compared with the 10%WG group. $^{\S\S}p < 0.01$ compared with the 10%WG group. $^{\#\#}p < 0.01$ compared with the corresponding sedentary group. $^{\#\#\#}p < 0.001$ compared with the corresponding sedentary group. $^{\dagger\dagger}p < 0.01$ compared with the NA + Ex group. $^{\dagger\dagger\dagger}p < 0.001$ compared with the NA + Ex group.

4.3.7. Quantitative Analysis of Inflammation-Related Plasma Cytokine Levels

As shown in Figure 18, the plasma cytokine levels were quantified by flow cytometry to confirm whether systemic cytokines were altered in response to the topical application of two concentrations of wintergreen oil. The plasma levels of IL-1β, IL-10, IL-27, IFN-β, GM-CSF, and MCP-1 were not significantly changed among all groups. In contrast, the plasma levels of IL-1α, IL-6, and TNF-α in the WG group were significantly higher than those in the NA and 10%WG groups. In addition, similar to results in the sedentary groups, the plasma levels of IL-1α, IL-6, and TNF-α in the WG + Ex group were also significantly elevated compared with those in the NA + Ex and 10%WG + Ex groups.

The plasma levels of IL-17A were only significantly elevated in the WG + Ex group compared with those in the 10%WG + Ex group, but there were no other significant changes. There were no significant changes in the plasma levels of IFN-γ among sedentary groups. In contrast, the plasma levels of IFN-γ were significantly decreased in the NA + Ex group compared with those in the NA group. Furthermore, the plasma levels of IFN-γ in the WG + Ex group were significantly higher than those in the 10%WG + Ex group.



A
B
C
D
E
F
G
H
IL-1α (pg/ml)
IL-1β (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
IL-17A (pg/ml)
IL-27 (pg/ml)
IFN-β (pg/ml)
IFN-γ (pg/ml)
***, § § §
††, ¶
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NA
10%WG
WG
NA + Ex
10%WG + Ex
WG + Ex

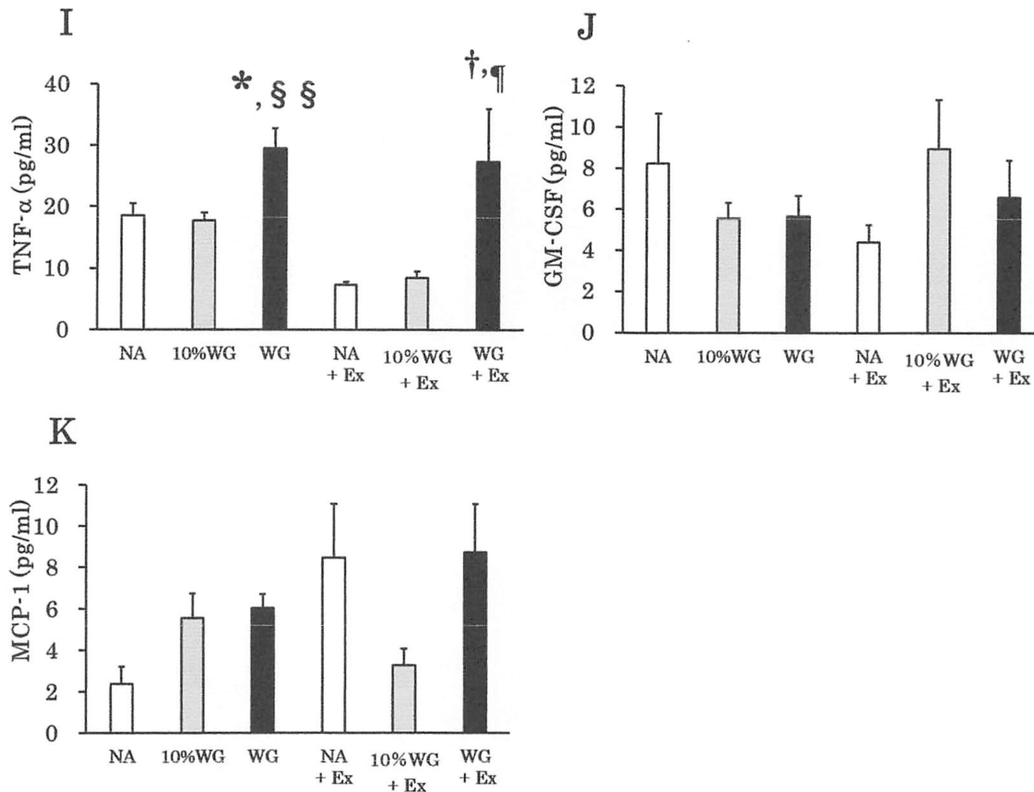

Figure 18. Plasma cytokine levels of (A) IL-1α, (B) IL-1β, (C) IL-6, (D) IL-10, (E) IL-17A, (F) IL-27, (G) IFN-β, (H) IFN-γ, (I) TNF-α, (J) GM-CSF, and (K) MCP-1, as measured by flow cytometric analysis. NA, the naïve control group. 10%WG, the sedentary group with 10% wintergreen oil applied. WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied. IL, interleukin; IFN, interferon; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1. Values are means ± SE, n = 6–8 (WG group: n = 6). *$p < 0.05$ compared with the NA group. ***$p < 0.001$ compared with the NA group. §§$p < 0.01$ compared with the 10%WG group. §§§$p < 0.001$ compared with the 10%WG group. ##$p < 0.01$ compared with the corresponding sedentary group. †$p < 0.05$ compared with the NA + Ex group. ††$p < 0.01$ compared with the NA + Ex group. ¶$p < 0.05$ compared with the 10%WG + Ex group. ¶¶$p < 0.01$ compared with the 10%WG + Ex group.

4.3.8. Effects of Exhaustive Exercise With or Without the Topical Application of Wintergreen Oil on the Expression Levels of Beta-Oxidation-Related Genes in Skeletal Muscles

We next assessed the β-oxidation-related mRNA expression levels in skeletal

muscles to assess the effect of the topical application of wintergreen oil on treadmill running in mice. The expression levels of *Cpt-1a* in the soleus muscle were significantly lower in the WG group than in the NA group (Figure 19 A). In contrast, no significant differences in the expression levels of *Cpt-1a* in the gastrocnemius muscle were observed among all groups (Figure 19 B).

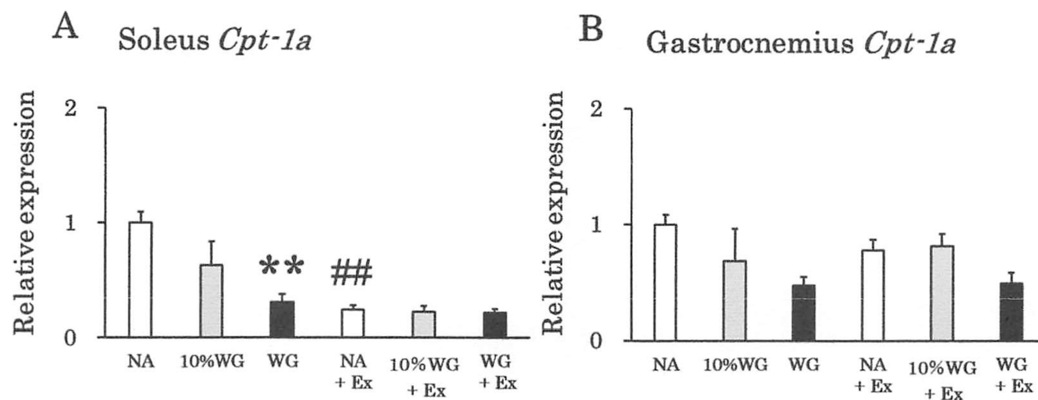

Figure 19. Gene expression levels of *Cpt-1a* in the soleus and the gastrocnemius muscle. NA, the naïve control group; 10%WG, the sedentary group with 10% wintergreen oil applied; WG, the sedentary group with undiluted wintergreen oil applied; NA + Ex, the exercise group with no oil applied; 10%WG + Ex, the exercise group with 10% wintergreen oil applied; WG + Ex, the exercise group with undiluted wintergreen oil applied; *Cpt-1a*, carnitine palmitoyl transferase-1α. Values are means ± SE, n = 6–8 (WG group: n = 6). $^{**}p < 0.01$ compared with the NA group. $^{\#\#}p < 0.01$ compared with the corresponding sedentary group.

4.4. Discussion

4.4.1. Wintergreen Oil Suppresses Exercise-Induced Inflammatory Cytokines (Il-1b and Il-6) in the Soleus Muscles at the Transcriptional Level

The purpose of the present study was to confirm whether two different concentrations of wintergreen oil, applied before exercise, could alleviate exercise-induced inflammation. The safety of transdermal absorption of wintergreen oil, which is sometimes used as an undiluted solution, was evaluated using blood biochemical data

and the run time-to-exhaustion based on treadmill running. In this study, we also measured the gene expression levels of *Il-1* and *Il-6* as pro-inflammatory cytokines and *Il-1ra* and *Il-10* as anti-inflammatory cytokines. Since inducible NO synthase (iNOS) is induced by pro-inflammatory cytokines in the inflammatory zone and causes cytotoxicity, we also assessed its expression levels. First, we examined the effects of exercise itself on the gene expression of pro-inflammatory cytokines by comparing the NA group versus the NA + Ex group. The expression levels of *Il-1b* and *Il-6* in the soleus muscle and liver, as well as *Il-1b* in the gastrocnemius muscle, were significantly increased by exhaustive exercise. However, the expression levels of *Il-1b* and *Il-6* in the heart were not significantly increased by exhaustive exercise. Next, we determined whether this exercise-induced increase in the expression of pro-inflammatory cytokine genes was suppressed by wintergreen oil. The results showed that the gene expression levels of *Il-1* and *Il-6* were significantly suppressed only in the soleus muscles with both concentrations of wintergreen oil. A possible reason for this is that blood flow to active muscles increases during exercise (117), and transdermally absorbed methyl salicylate could easily reach to the soleus muscles involved in endurance exercise and exert its anti-inflammatory effect. The cause of the anti-inflammatory effect of wintergreen oil observed only in the active muscles may be partially related to the transdermally absorbed compound that perfuses into the organ without a first-pass effect in the liver. In addition, because blood flow to the digestive system is reduced during exercise, the gene expression levels of pro-inflammatory cytokines in the liver may not have been suppressed by the topical application of wintergreen oil. The inhibitory effect of methyl salicylate on exercise-induced pro-inflammatory cytokine gene expression was not significantly different between the 10%WG + Ex and the WG + Ex groups (Figures 14-

17). These results indicate that a concentration of at least 10% wintergreen oil can suppress local exercise-induced muscle inflammation at the gene expression level.

However, a possible mechanism for the anti-inflammatory effect of methyl salicylate was not only a decrease in the expression levels of pro-inflammatory cytokines, but also an increase in the gene expression levels of anti-inflammatory cytokines. Therefore, we analyzed the expression levels of *Il-1ra* and *Il-10* as anti-inflammatory cytokines. IL-1ra is a cytokine that inhibits IL-1b signaling (110), whereas IL-10 is also an anti-inflammatory cytokine known to inhibit the synthesis of pro-inflammatory cytokines, such as TNF-α (111). Comparing the effects of exercise itself on anti-inflammatory cytokines between the NA and NA + Ex groups, we found a significant increase in *Il-10* expression levels in the soleus muscle and the heart, as well as a significant increase in the gene expression of *Il-1ra* and *Il-10* in the liver. We then determined whether this exercise-induced increase in anti-inflammatory cytokine gene expression was suppressed by wintergreen oil by comparing the exercise groups. The results showed that *Il-10* expression levels were significantly lower in both the soleus muscle and heart in both the 10%WG + Ex and WG + Ex groups compared with those in the NA + Ex group. The gene expression level of *Il-10* in the liver was significantly lower than that in the NA + Ex group only in the WG + Ex group with a high methyl salicylate concentration. The reason for the reduced expression of *Il-10* may be that with respect to the soleus, *Il-10* gene expression is associated with increased levels of *Il-6* expression (103). However, in the liver, heart, and gastrocnemius muscles, there is no linked variation in the expression levels of *Il-6* and *Il-10*, suggesting a pathway different than the induction of *Il-10* expression by *Il-6*. However, when the effects of exercise itself on the plasma levels of cytokines in the NA and NA + Ex groups were compared using flow

cytometry, no inflammatory cytokines were significantly increased after exercise. IL-6 is a myokine that is secreted with muscle contraction (115), and blood levels are generally known to increase after exercise. The plasma levels of IL-6 in the NA + Ex group did not increase compared with those in the NA group in the present study, which may be attributed to the statistical analysis performed using multiple comparisons between multiple groups. In fact, in a four-group treadmill running experiment conducted with a similar protocol in our laboratory, there was a significant increase in the plasma levels of IL-6 in the NA + Ex group compared with those in the NA group (in submission).

4.4.2. Topical Application of Undiluted Wintergreen Oil Before Exercise Reduces Running Time by Inhibiting Beta-Oxidation in the Soleus Muscles and Elevates Both Local and Systemic Inflammation

This study revealed that 10% wintergreen oil, applied to mice 30 min before treadmill running, significantly shortened the run time-to-exhaustion compared with that in the NA + Ex group (Figure 12). More importantly, undiluted wintergreen oil applied 30 min before treadmill running resulted in a more significant reduction in time-to-exhaustion compared with that in the NA + Ex group (Figure 12). There are some possible reasons for the significant decrease in the run time-to-exhaustion in the WG + Ex group. First, significant elevations in the gene expression levels of pro-inflammatory cytokines (*Il-1b* and *Il-6*) were observed in the WG group in the gastrocnemius muscle, heart, and liver, compared with those in the other sedentary groups (Figure 14). The gene expression levels of pro-inflammatory cytokines were also increased in the liver and heart at rest because the auto-regulation of blood flow (approximately 25% and 5% of cardiac output, respectively) may have contributed to the increased exposure to methyl

salicylate. Additionally, the plasma levels of pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α) were all significantly increased in the WG group compared with those in the NA and 10%WG groups (Figure 18). In contrast, regarding IL-10, an anti-inflammatory cytokine, its mean level in both 10%WG and WG groups increased relative to that in the NA group, but the difference was not statistically significant. These results suggest that the balance of pro-inflammatory and anti-inflammatory cytokines in the blood was skewed toward inflammation and that systemic inflammation was probably induced in the WG group at the start of the run. Since the timing of blood collection in the sedentary group was not the start of the run (30 min after topical application), additional experiments are needed to further investigate such results.

Regarding the metabolic changes in skeletal muscle due to exercise load, glucose is the main source of energy during high-intensity, short-duration exercise, whereas lipids (triglycerides and free fatty acids in the muscle) act as the main energy source during low- to moderate-intensity, long-duration exercise (151, 152). In addition, fatty acids are metabolized by β-oxidation in the mitochondria. To investigate whether transdermally administered wintergreen oil affects fatty acid metabolism, this study analyzed the gene expression of *Cpt-1a*, encoding the rate-limiting enzyme for β-oxidation (Figure 19). The expression levels in the soleus muscle were significantly lower in the WG group than in the NA group. However, there was no significant change in the expression levels of *Cpt-1a* in the soleus muscle between the 10%WG and NA groups. Meanwhile, there were no significant differences among all groups in the expression levels of *Cpt-1a* in the gastrocnemius muscle. The results of gene expression analysis of *Cpt-1a* in the soleus muscles comparing the three sedentary groups were consistent with the run time results in the corresponding exercise groups (Figure 12). This suggests that β-oxidation in the

slow-twitch muscle affects run time-to-exhaustion for exhaustive exercise. Salicylic acid poisoning has been reported to affect lipid metabolism (45), and the topical application of undiluted wintergreen oil may have affected the expression of *Cpt-1a*. This study also analyzed gene expression in the soleus muscle, in which slow-twitch fibers account for approximately 87%, and the gastrocnemius muscle, in which fast-twitch fibers account for approximately 93% (153). In endurance exercise, the depletion of glycogen in slow-twitch muscles and some fast-twitch muscles is associated with muscle fatigue. Fast-twitch muscles have a high capacity to produce adenosine triphosphate (ATP) anaerobically through glycolytic reactions and can contract quickly. In contrast, slow-twitch muscles produce energy primarily through aerobic pathways. The aerobic ATP-producing capacity of slow-twitch muscles is related to their high number of mitochondria and high enzymatic activity for lipid metabolism.

Methyl salicylate, the main component of wintergreen oil, is metabolized by the liver to salicylate; however, first-pass metabolism also occurs in the skin, and esterases rapidly hydrolyze salicylates to release active salicylate in both the epidermis and dermis (145, 146, 154, 155). However, the activity of methyl salicylate hydrolysis to salicylate per protein weight is two orders of magnitude higher in the liver than in the skin (154). Importantly, salicylate has been reported to inhibit β-oxidation in rodents (156-158) and humans (159). These results suggest that methyl salicylate was metabolized to salicylate in the WG group, and this salicylate may have inhibited β-oxidation via the repression of *Cpt-1* gene expression in the mitochondria-rich slow-twitch muscle (soleus muscle). As a result, run time-to-exhaustion may have been significantly shortened due to the suppression of lipid metabolism, which is important as an energy substrate during prolonged exercise, and the early depletion of muscle glycogen (160).

Meanwhile, biochemical data (Figure 13) suggested several reasons for the shortened run time-to-exhaustion in the WG group. Elevated levels of ALT, AST, and ALP were observed in the WG group as enzymes of the hepatobiliary system (Figure 13). AST is abundant in the myocardium, liver, skeletal muscle, and kidneys, but is only present in trace amounts in the blood. Therefore, elevated plasma AST levels reflect cellular degeneration and necrosis in these organs. In contrast, ALT is abundant in the liver, kidney, myocardium, and skeletal muscle, but its content is lower than that of AST. Even though ALT is most abundant in the liver, its levels are approximately one-third those of AST. In particular, elevated plasma ALT levels are reflective of the degeneration and necrosis of hepatocytes. ALP is markedly elevated in biliary enzymes during acute drug-induced liver injury of the cholestasis type, whereas ALP is only mildly elevated in hepatitis. In the present study as well, ALP levels were only mildly elevated in the WG group compared with ALT and AST levels, suggesting that the changes in biochemical data reflect liver injury. The increase in transaminases (AST and ALT) is consistent with that reported for salicylate poisoning in humans (161).

We also measured BUN and CRE, which are commonly used in renal function tests, and found a significant increase in plasma levels of BUN and a significant decrease in plasma levels of CRE in the sedentary groups. BUN is a measurement of nitrogen and urea in the blood. Urea is the final metabolite of ingested protein and tissue breakdown products, and after being filtered in the glomeruli of the kidney, approximately 50% is reabsorbed in the tubules, whereas the rest is excreted in the urine. Therefore, urea is affected by decreased kidney function and increased protein catabolism, as well as the urine volume. Meanwhile, CRE, another marker of renal function, is hardly affected by extra-renal factors, and the BUN/CRE ratio is used to estimate the degree of influence

of extra-renal factors. Therefore, the significantly elevated BUN/CRE ratio in the WG group suggests that extrarenal factors were responsible for the elevated plasma BUN concentration. Since CRE is a metabolized form of creatine produced in the liver, the decrease in creatine production in the liver was thought to be the cause of the decrease in plasma CRE concentrations. In this study, plasma levels of ALT and AST were significantly increased in the WG group, suggesting that salicylic acid was responsible for liver damage. Furthermore, the expression levels of *Cpt-1a* were significantly suppressed in the WG group, suggesting that the energy substrate had shifted to protein and carbohydrates as a result of the suppression of lipid metabolism. This finding is supported by the decrease in the plasma levels of GLU and the increase in the plasma levels of LA in the WG group. These results also suggest that the increase in plasma BUN concentrations in the WG group was caused by hepatotoxicity and increased protein catabolism caused by salicylic acid. These changes in biochemical data may have occurred in the WG group before the run and could be one of the factors that significantly shortened the run time-to-exhaustion in the WG + Ex group. Salicylic acid poisoning can cause rhabdomyolysis and an increase in the plasma levels of CK, but no such increase in CK and no overdose-induced muscle damage were observed in the present study. CK is an enzyme that is distributed in the skeletal muscle, cardiac muscle, smooth muscle, and the brain and is involved in the marked increase in high-energy phosphate binding and the reproduction of ATP. Because CK is largely absent in the blood cells and liver, the increase in plasma CRE reflects cellular injury in these tissues. Although muscle injury can increase CRE and CK, we did not find a significant increase in these parameters in the WG group. However, the increase in BUN is consistent with that reported in salicylate poisoning (162). Therefore, when wintergreen oil is used in sports

aromatherapy massage for its anti-inflammatory properties, it should be used with caution to avoid overdosing for safety reasons.

In addition, biochemical data from the exercise group showed that ALT and AST levels were significantly higher in the WG + Ex group than in the other exercise groups, but were significantly lower compared with the WG group (Figure 13). This result may be related to reduced exposure to salicylic acid due to reduced blood flow to the liver during exercise. However, the application of undiluted wintergreen oil before exercise impaired endurance capacity (Figure 12). Therefore, we examined whether the wintergreen oil at a 10% concentration could improve the biochemical data that were negatively affected by exhaustive exercise, and found that BUN was significantly lower in the 10%WG + Ex group than in the NA + Ex group. This suggests that wintergreen oil at a 10% concentration inhibited exercise-induced acute kidney injury.

Acute liver injury, a decrease in the expression level of the *Cpt-1a* gene involved in beta-oxidation, and the induction of systemic inflammation may be the mechanism through which wintergreen oil application decreased the endurance capacity. Wintergreen oil diluted to 10% with jojoba oil can be used prophylactically to mitigate exercise-induced inflammation, but it should be diluted with a carrier oil for safety reasons. This is especially true when applied extensively. The results of this study suggest that the methyl salicylate preparations should not be used extensively or in high doses prior to endurance exercise because its high concentrations can inhibit oxidation. The safety of aromatherapy massage to athletes can be ensured by considering the optimal concentration of essential oils and their optimal combination with carrier oils to prevent undesirable side effects while reducing exercise-induced inflammation. Finally, the transdermal absorption of methyl salicylate is significantly enhanced in a hot

environment (40°C outside) with and without exercise (163). Therefore, for safety reasons, further diluting the wintergreen oil is necessary when using it before and after exercise in hot environments.

4.5. Conclusions

The topical administration of 4 μL of 10% (v/v) wintergreen oil per gram of body weight to the dorsal skin of mice 30 min before treadmill running in the 10%WG + Ex group significantly decreased the endurance capacity compared with that in the NA + Ex group. Additionally, the WG + Ex group, which was administered undiluted wintergreen oil, had a much shorter run time-to-exhaustion than the two other exercise groups. In the WG group, the application of undiluted wintergreen oil elicited acute hepatotoxicity, and *Cpt-1*, which is involved in the metabolism of fatty acids, was repressed at the gene expression level. Plasma inflammatory cytokine levels increased and systemic inflammation was induced. These mechanisms may have reduced the time to exhaustion on the treadmill in the WG + Ex group.

In the 10%WG + Ex group, there was no significant change in plasma cytokines. However, the gene expression of pro-inflammatory cytokines was significantly lower in the soleus muscle and liver than in the NA + Ex group. These results suggest that the topical wintergreen (methyl salicylate) is transported by the bloodstream to the active muscle to alleviate exercise-induced inflammation by suppressing local gene expression levels of pro-inflammatory cytokines in the muscle. In addition, the 10%WG + Ex group had lower plasma levels of BUN than the NA + Ex group, suggesting that exercise-induced renal injury was suppressed.

Application of wintergreen oil diluted to 10% on the entire back had a beneficial

anti-inflammatory effect on active muscles, but it also had an unfavorable result of decreasing the endurance capacity. Although the amount of massage oil used in human massage varies considerably depending on the density of hair and body size, when the amount applied in this study was converted to the amount used in humans, it was close to the upper limit used in a full-body massage. Given the large amount of oil applied, the amount of methyl salicylate administered as salicylate increased even with a low concentration of wintergreen oil, which may have decreased the endurance capacity. Therefore, extensive application of methyl salicylate-containing poultices just before exercise should be avoided.

In summary, athletes should avoid the excessive use of methyl salicylate-containing preparations or wintergreen oil in sports massage. The optimal dose of wintergreen oil that can be used to prevent or alleviate exercise-induced inflammation should be considered in future studies.

Chapter 5.

Conclusions

5.1. New Scientific Findings from a Series of Experiments

In recent years, sports massage before exercise has been attracting attention for its application in the prevention of sports injuries and conditioning. Sports aromatherapy massage before exercise is considered to be essential for improving muscular movement because it has the advantage of being effective even in a short time with the help of components contained in essential oils. Although Jojoba oil is widely used as a carrier oil to dilute essential oils, limited information is available on its effects on blood biochemical parameters. Therefore, in the first study, we confirmed how the components of jojoba oil absorbed through the skin affect plasma biochemical parameters in mice. As a result, it was revealed that plasma NEFA levels were increased by approximately 20% in 30 min after the topical application of jojoba oil in mice (98). To investigate the underlying mechanism, we further analyzed the expression levels of lipid metabolism-related genes in various tissues/organs after the topical application of jojoba oil. As a result, the elevated plasma NEFA levels might have been caused of both enhanced lipolysis via *Atgl* upregulation in the liver and reduced fatty acid trafficking via *FABPpm*, *FATP1*, *FATP3*, and *FATP4* downregulation in the skin.

Jojoba oil has been reported to have anti-inflammatory properties (55, 96). Meanwhile, it is well known that lipid utilization increases during endurance exercise in active muscle (97). Therefore, in the second study, we investigated whether the topical

application of jojoba oil at 30 min before treadmill running could prevent exercise-induced inflammation and to evaluate whether the topical application of jojoba oil affects the run time-to-exhaustion. As a result, the topical application of jojoba oil 30 min before treadmill running did not affect endurance capacity in mice. Regarding exhaustive exercise-induced inflammation, the topical application of jojoba oil 30 min before treadmill running significantly decreased some pro-inflammatory cytokines (*Il-1β* and *Il-6*) at the mRNA level in skeletal muscles, and especially, in the slow-twitch muscle, which contributes to endurance capacity. However, there were no significant changes in plasma cytokine levels due to the topical application of jojoba oil among sedentary groups. In summary, topical application of jojoba oil, when applied 30 min prior to strenuous exercise, might suppress exercise-induced inflammation in muscles at the gene expression level.

Several essential oils have been reported to have anti-inflammatory and antioxidative effects (28-33, 55). For example, wintergreen oil, which contains high concentrations (>99%) of methyl salicylate, is metabolized to salicylate after transdermal absorption and exerts anti-inflammatory effects in the body (145, 146). In fact, methyl salicylate has been used by many people as an active ingredient in poultices and other active ingredients to relieve musculoskeletal pain (147). Therefore, in the third study, we investigated whether the wintergreen oil (undiluted wintergreen oil and wintergreen oil diluted to 10% with jojoba oil) applied 30 min before exercise can alleviate exercise-induced inflammation. In addition, this study measured the run time-to-exhaustion in treadmill running. Since wintergreen oil is sometimes used as undiluted solutions, this study also evaluated the safety of the transdermal absorption of wintergreen oil using blood biochemical data. As a result, topical application of undiluted wintergreen oil

significantly reduced exercise endurance capacity. The mechanism underlying this adverse effect was investigated using data from the resting groups. In the WG group, severe acute liver injury was induced. Additionally, *Cpt-1*, which is involved in β-oxidation in the slow-twitch muscle, was suppressed, and the level of plasma inflammatory cytokines increased. Application of 10% wintergreen oil also significantly shortened the running time, but significantly decreased the gene expression of inflammatory cytokines in the soleus muscle and liver compared with the NA + Ex group. Thus, the application of 10% wintergreen oil suppressed the gene expression level of inflammatory cytokines in muscle. However, the use of wintergreen oil should be further investigated to benefit from its favorable effects without developing adverse events, such as liver damage. As compared to the NA + Ex group, plasma BUN levels immediately after exercise were significantly reduced in the 10% WG + Ex group, suggesting that exercise-induced renal impairment might be suppressed. However, since exercise-induced renal injury (plasma levels of BUN) was not suppressed in the JO + Ex group as compared to the NA + Ex group, the effect seen in the 10% WG + Ex group might be due to the effect of methyl salicylate in wintergreen oil rather than the synergistic effect of jojoba oil as a solvent. In fact, the WG +Ex group, in which undiluted wintergreen oil without jojoba oil was applied, also had significantly lower plasma levels of BUN immediately after exhaustion.

5.2. Implications for Future Research

The present study revealed that both wintergreen oil at a 10% concentration and jojoba oil reduced exercise-induced inflammation in active muscles at the gene expression level. However, DOMS usually occurs within the first 24 h after exhaustive

exercise and has been reported to peak between 24 and 72 h (92). Thus, it is necessary to study the effects of the drug not only immediately after exercise, but also 24 or 72 h after exercise. Studies with hydrocortisone and methyl salicylate have also shown that the absorption rate of drugs varies greatly depending on the site of application (148, 164). In addition, transdermal absorption is affected by the distribution of blood vessels, temperature, and density of skin appendages such as hair follicles and sweat glands. Therefore, to develop sports aromatherapy massage on a scientific level, it is essential to examine the optimal dosage and concentration for each site and validate its efficacy and safety through studies using human subjects.

In the current study, wintergreen oil was diluted to 10% with jojoba oil. As mentioned earlier, it is also important to determine the optimal dose and dosage of wintergreen oil, taking into account the balance between anti-inflammatory effects and side effects. The current study also suggests that jojoba oil can alleviate exercise-induced inflammation when used as a carrier oil. Although several essential oils have been identified as having anti-inflammatory properties (28-33, 55), this is the first report to examine the effects of carrier oil and essential oil on exercise-induced inflammation. In the future, it will be important to examine the synergistic effects of essential oils and carrier oils and to verify the effects of essential oils and carrier oils on exercise-induced inflammation in clinical trials.

Chapter 6.

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Chapter 7.

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