Effects of Exposure to Mild Hyperbaric Oxygen on Skeletal Muscle Fibers, Epidermal Basal Cells, and Skin Pigmentation

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Abstract

The effects of exposure to mild hyperbaric oxygen on skeletal muscle fibers, epidermal basal cells, and skin pigmentation in mice and humans at different ages were investigated. Five-, 34-, 55-, and 88-week-old mice were exposed to mild hyperbaric condition at 1.25 atmospheres absolute (ATA) with 36% oxygen for 6 h per day for 2 weeks. The properties of the skeletal muscle in mice, which were exposed to mild hyperbaric oxygen, were compared with those in age-matched mice under normobaric conditions (1 ATA with 21% oxygen). The activity of succinate dehydrogenase (SDH), which is a mitochondrial oxidative marker enzyme, in the tibialis anterior muscle of normobaric mice decreased with age. The decreased SDH activity was also observed in both type IIA and type IIB fibers in the muscle of normobaric mice at 57 and 90 weeks. In contrast, the decreased SDH activity of the muscle and fibers was recovered in hyperbaric mice at 57 and 90 weeks. It is suggested that exposure to mild hyperbaric oxygen used in this study reduces the age-related decrease in the oxidative capacity of skeletal muscles (conclusion obtained from the study of Chapter 2). Thickness and numbers of epidermal basal cells on the back skin of 5-, 34-, 55-, and 88-week-old mice, which were exposed to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen, were compared with those in age-matched mice under normobaric conditions. The number and proliferative activity of epidermal basal cells increased after 1 week and 2 weeks of exposure to mild hyperbaric oxygen, irrespective of the age. It is suggested that exposure to mild hyperbaric oxygen used in this study accelerates the proliferative activity of epidermal basal cells in the mouse skin, irrespective of the age (conclusion obtained from the study of Chapter 3). The effects of exposure to mild hyperbaric oxygen on ultraviolet B (UVB) irradiation-induced melanin pigmentation of skins and senile spot sizes of faces were investigated. In the 1st experiment, male subjects were irradiated with UVB on their upper arms for inducing erythema and the subsequent melanin pigmentation. Thereafter, they were exposed to mild hyperbaric oxygen at 1.25 ATA with 32% oxygen for 1 h per day, 3 times per week. Melanin pigmentation induced by UVB-irradiation lightened after 4 weeks of exposure to mild hyperbaric oxygen. In the 2nd experiment, female subjects were exposed to mild hyperbaric oxygen for 1 h per day, 2 times per week. Senile spot sizes became small after 12 weeks

of exposure to mild hyperbaric oxygen at 1.25 ATA with 32%. Exposure to mild hyperbaric oxygen accelerated both the fading in melanin pigmentation and the decrease in senile spot size. It is suggested that mild hyperbaric oxygen used in this study is effective for melanin pigmentation of skins and senile spot sizes of faces (conclusion obtained from the study of Chapter 4). Exposure to mild hyperbaric oxygen at 1.25 ATA with 32-36% oxygen is effective for maintaining and improvement on skeletal muscle fibers, epidermal basal cells, and skin pigmentation.

Chapter 1
General Introduction

For mammals to survive in terrestrial environments, the loss of water from inside of the body must be paid special attention. To keep the water inside our body, the mammals have a stratum corneum (SC) at the outermost layer of skin epidermis [81]. The SC is multilayered organization which is constructed by corneocytes and intercellular lipids (ICL). In general, the SC has been likened to brick wall, in which is hydrophilic corneocytes (bricks), is embedded within a hydrophobic lipid (mortar). These heterogeneous structures stack 6-86 layers and are constructed 15 to 30 μm thickness, and it enables selective permeability, water barrier function, and protect against various external stimulus as dryness, friction, sebum, sweat, wand ash by surfactant, ultra violet, and other factors [88]. However, the number and thickness of these layers in SC are topographical variations depending on body site and mechanical stressors [103].

It is widely known that dryness of external environment leads SC to inflexible [82] and to thick [13]. In addition, it is also reported that ultraviolet (UV) rays reduce barrier function of SC because of disrupting ICL structure [56], free fatty acid which derives from sebum induces rough skin [45], and sweat reduces barrier function of SC [89]. Thus, stratum corneum has always been damaged by various external stimuli. Therefore, the SC which was damaged comes off spontaneously, so that new SC needs to be constructed [84]. In the human cheek, the full biological period required for complete regeneration of epidermis is estimated an average of 28-30 days [57]. These regeneration processes of skin are called “turn-over.” This renewal of skin is supported by proliferation of epidermal cells at the basal cell layer. The divided epidermal cells differentiate into keratinocytes at spinous and granular cell layers, and SC is completed for about 2 weeks.

However, the proliferative rate of keratinocyte, which is cultured, declines with age [20]. In addition, it is reported that epidermal growth factor-stimulated DNA synthesis ability markedly decreases on hepatocytes in rats with age [34]. Therefore, the delay and reduction of DNA synthesis have reported on rat liver [95]. In skin organization, proliferation activity of epidermal cells decreases with age, and turn-over speed of aged

skin becomes lower [84]. As the results, epidermal thickness becomes thin, re-epithelization after wound healing delays [20, 87], and senescent keratinocyte accumulates with age [5]. Therefore, it is considered that aged skin decreases elasticity [17, 83].

To improve external appearance and physical properties of skin with age, activation of epidermal cells is one of the key factors. In the field of beauty, one of the methods to enhance epidermal cell activity by artificially, chemical peeling is often used to improve photo-aged skin such as wrinkles and pigmentation spots [58]. Keratinocytes release IL1-arfa which is inflammatory cytokine by chemical peeling treatment, so that caused inflammatory reaction forces keratinocyte to accelerate proliferation activity [104]. On the other hand, it is reported that proliferation speed of keratinocyte on psoriasis skin which observes severe dry symptom is faster than normal skin, because activation of T lymphocytes leading to release of cytokines causes proliferation of keratinocytes [50]. These results indicate that proliferation activity of keratinocyte plays an important role to keep skin beauty and healthy, but if we try to regulate the proliferation speed in aged skin, we should pay much attention to safety.

It is considered that oxygenation is a rational process to increase proliferation activity of keratinocytes and may bring a beneficial result to improve aged skin symptoms. This is because the efficacy of high oxygen concentration has been reported as the method of cutaneous wound-healing process [44]. They confirmed that hyperbaric oxygen treatment (the pressure was increased to 2.4 atmospheres absolute (ATA) and the conditions were maintained for 90 minutes) accelerates the reconstruction of epidermis, and the increase of p63 which is a maker of undifferentiated proliferating cell. These results suggested that oxygen supply to epidermis has a possibility to activate proliferation activity of keratinocytes.

In general, patients are exposed clinically to hyperbaric conditions of 2-3 ATA with 100% oxygen as hyperbaric oxygen therapy [49, 93]. Hyperbaric oxygen therapy leads to vasoconstriction and hyperoxygenation, making it an effective treatment for patients with various clinical disorders such as severe carbon monoxide poisoning, decompression sickness, and arterial gas embolism and as adjunctive therapy for the prevention and treatment of osteoradionecrosis, clostridial myonecrosis, and compromised skin grafts and flaps [4, 64, 91, 97].

However, oxygen therapy with or without pressure is associated with the risk of oxygen toxicity and excessive oxidative stress. Oxidative stress plays as key role in the pathogenesis of many diseases and their complications; the generation of free radicals and increased levels of oxidative stress are associated with atherosclerosis, cataract, retinopathy, myocardial infarction, hypertension, diabetes, renal failure, and uremia [12, 22, 53].

In previous studies using experimental animals [19, 77] and humans [18, 78], exposure to hyperbaric oxygen has the potential to induce and accelerate myopia and cataract. Furthermore, a previous study [19] reported that cataracts in 17- to 18-month-old guinea pigs were induced by exposure to hyperbaric conditions at 2.5 ATA with 100% oxygen for 2 to 2.5 h, 3 times per week, up to 100 times. Similarly, myopia and cataracts developed in human lenses after exposure to prolonged hyperbaric conditions at 2 to 2.5 ATA with 100% oxygen for 1.5 h, once per day, from 150 to 850 times [78] but were observed rarely after only 48 times [18]. Therefore, exposure to hyperbaric conditions at 2–3 ATA with 100% oxygen has the potential to induce and accelerate myopia and cataracts. In addition, hyperbaric oxygen therapy is considered to cause excessive production of reactive oxygen species in several tissues and organs [75, 76], suggesting that oxidative stress induced by hyperbaric oxygen therapy accelerates tissue damage. Oxidative stress levels depend not only on the pressure but also on the duration of the exposure to hyperbaric oxygen; the pressure of exposure from 2.5 to 3 ATA and the duration of exposure from 90 to 120 min result in a pronounced increase in oxidative stress level of rats [75, 76].

Exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen is used to improve metabolism without enhanced levels of oxidative stress. A mild hyperbaric oxygen chamber has been developed; the chamber consists of an oxygen tank with an oxygen concentrator and an air compressor that automatically maintain an elevated atmospheric pressure and an increased oxygen concentration by using a computer-assisted system (Fig. 1).

It is reported that exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen enhances the oxidative enzyme activity of cells and tissues [38, 55], therefore inducing inhibition in the growth-associated increase in blood glucose level of type 2 diabetic Goto-Kakizaki rats [24, 105, 107] and in blood pressure level of spontaneously

hypertensive rats [61]. These studies [24, 61, 105, 107] concluded that exposure to mild hyperbaric oxygen has a beneficial effect on oxidative metabolism in cells and tissues although the amount of dissolved oxygen is greater by exposure to hyperbaric exposure at 2-3 ATA with 100% oxygen compared with that at 1.25 ATA with 36% oxygen.

Fig. 1. Mild hyperbaric oxygen chambers for experimental animals (right) and humans (left). These chambers consist of an oxygen tank with an oxygen concentrator and an air compressor that automatically maintain an elevated atmospheric pressure and an increased oxygen concentration by using a computer-assisted system.

In this study, in order to verify the efficacy of exposure to mild hyperbaric oxygen on the age-related skeletal muscle and skin, 3 points were focused as follows: 1) the oxidative enzyme activity of skeletal muscles in mice at different ages was examined to confirm whether mild hyperbaric oxygen has effects on age-related changes of aerobic metabolism, 2) the proliferation activity of keratinocytes in mice at different ages was examined to verify the efficacy of exposure to mild hyperbaric oxygen on skin, and 3) human senile spot sizes, which indicate skin troubles with age, were investigated whether exposure to mild hyperbaric oxygen improves senile spot sizes.

Chapter 2

Exposure to Mild Hyperbaric Oxygen Reduces Age-related Decrease in Oxidative Capacity of the Tibialis Anterior Muscle in Mice

2-1. Introduction

A reduction in skeletal muscle mass is one of the most striking features of the aging process. Previous studies [6, 30, 51] have indicated that this reduction is due to decreases in the number and volume of individual fibers in skeletal muscles. Mammalian skeletal muscles consist of different sizes and types of fibers, for example, slow-twitch type I and fast-twitch type II fibers [65, 66]. A reduction in the number and volume of type II fibers in skeletal muscles of rats can be observed in the initial stages of the aging process [35, 39]. These changes in type II fibers are considered to be due to a transition of fiber types from type II to type I, selective loss and atrophy of type II fibers, and/or degeneration in the neuromuscular junction, which are induced by age-related disuse of type II fibers. A decrease in the number and volume of both type I and type II fibers in skeletal muscles of rats can be observed in the late stages of the aging process [28, 35, 39]. These changes in type I and type II fibers are closely related to the loss and degeneration of spinal motoneurons innervating those fibers in skeletal muscles. Furthermore, a decrease in the oxidative enzyme activity of skeletal muscles in rats was observed with increasing age [2, 7, 48].

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma. An increase in both atmospheric pressure and oxygen concentration enhances oxidative enzyme activity in mitochondria and consequently increases the oxidative metabolism in cells and tissues [38]; thus, it is expected that exposure to hyperbaric oxygen facilitates the turnover of oxidative metabolism, particularly of pathways in the mitochondrial TCA cycle, thereby reducing the age-related decrease in the oxidative enzyme activity of muscle fibers. Previous studies [38, 55] observed that a pressure of 1.25 ATA and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism. This study examined the oxidative capacity of the tibialis anterior muscle in mice at different ages, which were exposed to 36% oxygen at1.25 ATA. Furthermore, the cross-sectional

areas and oxidative enzyme activities of fibers, which were type-defined by ATPase activity, in the muscle of mice were determined using a quantitative histochemical analysis.

2-2. Materials and methods

All experimental procedures, including animal care, were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Physiological Society. This study was also approved by the Institutional Animal Care Committee at Kyoto University, Japan.

2-2-1. Animal care and treatment

Five-, 34-, 55-, and 88-week-old female mice were used in this study. The mice (the hyperbaric group; n = 6 in each age group) were exposed to hyperbaric conditions at 1.25 ATA with 36% oxygen, which were automatically maintained by a computer-assisted system, in a hyperbaric chamber for 6 h (11:00 to 17:00) and were placed under normobaric conditions at 1 ATA with 21% oxygen for 18 h (17:00 to 11:00), while other mice (the normobaric group; n = 6 in each age group) were placed in a hyperbaric chamber under normobaric conditions for 24 h. The hyperbaric chamber was 90 cm in length and 80 cm in diameter; thus, it could simultaneously house a number of rats (up to 20 cages).

All mice were individually housed in same-sized cages in a room maintained under controlled 12-h light/dark cycles (lights switched off from 20:00 to 08:00) at a temperature of 22 ± 2°C with a relative humidity of 45%–65%. Food and water were provided ad libitum to all mice.

2-2-2. Tissue procedures

After 2 weeks of exposure to hyperbaric oxygen, the mice in the normobaric and hyperbaric groups were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The tibialis anterior muscles from both hind limbs were removed and cleaned of excess fat and connective tissue. Thereafter, the mice were sacrificed by an overdose of sodium pentobarbital.

The tibialis anterior muscles of the right side were quickly frozen in liquid nitrogen

for measurement of succinate dehydrogenase (SDH) activity. The SDH activity was determined according to the method of Cooperstein et al. [11]. Briefly, the muscles were homogenized using a glass tissue homogenizer with 5 volumes of ice-cold 0.3 M phosphate buffer, pH 7.4. Sodium succinate was added to yield a final concentration of 17 mM. The final concentrations of the components of the reaction mixture were as follows: sodium succinate 17 μM, sodium cyanide 1 mM, aluminum chloride 0.4 mM, and calcium chloride 0.4 mM. This reaction mixture was transferred to the spectrophotometer and the reduction of cytochrome *c* was followed by observing the increase in extinction at 550 nm. The SDH activity was calculated from the ferricytochrome *c* concentration and protein content.

The tibialis anterior muscles of the left side were pinned on a cork at their in vivo length and quickly frozen in isopentane cooled with liquid nitrogen. The mid-portion of the muscle was mounted on a specimen chuck using a Tissue Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan). Serial transverse sections (10-μm thickness) of the muscle on the chuck were cut in a cryostat maintained at –20°C. The serial sections were brought to room temperature, air-dried for 30 min, and incubated for ATPase activity following acid preincubation and for SDH activity [36, 67].

The ATPase activity was determined by the following procedure: (1) preincubation for 5 min at room temperature in 50 mM sodium acetate and 30 mM sodium barbital in distilled water, adjusted to pH 4.5 with HCl; (2) washing in 5 changes of distilled water; (3) incubation for 45 min at 37°C in 2.8 mM ATP, 50 mM $CaCl_2$, and 75 mM NaCl in distilled water, adjusted to pH 9.4 with NaOH; (4) washing in 5 changes of distilled water; (5) immersion for 3 min in 1% $CaCl_2$; (6) washing in 5 changes of distilled water; (7) immersion for 3 min in 2% $CoCl_2$; (8) washing in 5 changes of distilled water; (9) immersion for 1 min in 1% $(NH_4)_2S$; (10) washing in 5 changes of distilled water; and (11) dehydration in a graded series of ethanol, passed through xylene, and then cover slipped (Fig. 2).

Classification into 2 fiber types was based on staining intensities for ATPase activity: type IIA (positive intensity) and type IIB (negative intensity) [29].

The SDH activity was determined by incubation in a medium containing 0.9 mM 1-methoxyphenazine methylsulfate, 1.5 mM nitroblue tetrazolium, 5.6 mM ethylene-diaminetetraacetic acid disodium salt, and 48 mM succinate disodium salt (pH 7.6) in

100 mM phosphate buffer. The incubation time was 10 min; the changes in staining intensity in response to incubation reached a plateau after 10 min. The reaction was stopped by multiple washings with distilled water, dehydrated in a graded series of ethanol, passed through xylene, and cover slipped.

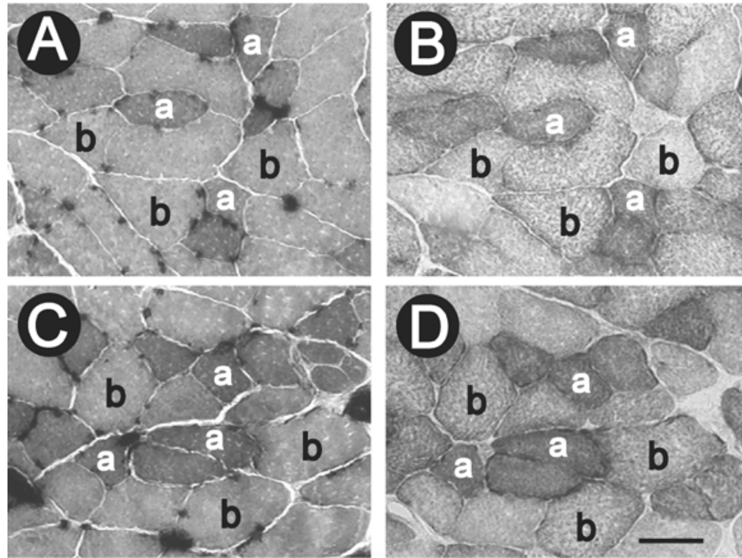

Fig. 2. Serial transverse sections of the tibialis anterior muscles in the normobaric (A and B) and hyperbaric (C and D) mice at 90 weeks. A and C, stained for ATPase activity following preincubation at pH 4.5; B and D, stained for succinate dehydrogenase activity. a, type IIA; b, type IIB. Scale bar = 50 μm.

The cross-sectional areas and SDH activities from approximately 300 fibers, which were type-defined by ATPase activity, in the central region of the muscle section were measured by tracing the outline of a fiber and stored in a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan) [62, 63]. The images were digitized as gray-level pictures. Each pixel was quantified as one of 256 gray levels that were then automatically converted to optical density (OD). A gray level of zero was equivalent to 100% transmission of light and that of 255 was equivalent of 0% transmission of light. The mean OD value of all pixels within a fiber was determined using a calibration tablet that had 21 gradient density steps and corresponding diffused density values.

2-2-3. Statistical analyses

The data were expressed as mean and standard deviation. One-way analysis of variance was used to evaluate the age-related changes. When the differences were found to be significant, further comparisons were made by performing *post hoc* tests. The differences between the normobaric and age-matched hyperbaric groups were determined by using the *t*-test. A probability level of 0.05 was considered to be statistically significant.

2-3. Results

2-3-1. Body weight

An age-related increase in body weight was observed in the normobaric groups; the body weights at 36 and 57 weeks were greater than that at 7 weeks, and the body weight at 90 weeks was the greatest among the groups (Fig. 3A). These results were similar in the hyperbaric groups.

There were no differences in body weight between the normobaric and age-matched hyperbaric groups, irrespective of the age.

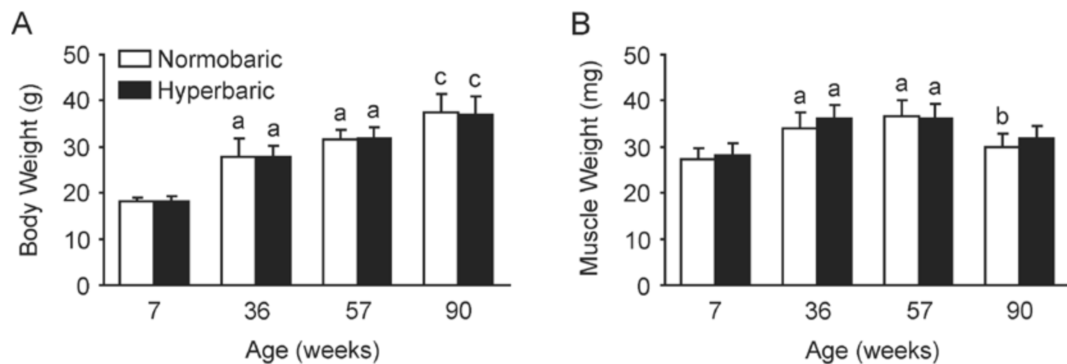

Fig. 3. Body weights (A) and tibialis anterior muscle weights (B) of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from 6 animals. The mice in the hyperbaric group were exposed to 1.25 ATA with 36% oxygen for 6 h per day for 2 weeks. $^{a}p < 0.05$ compared with the corresponding group at 7 weeks; $^{b}p < 0.05$ compared with the corresponding groups at 7, 36, and 57 weeks; $^{c}p < 0.05$ compared with the corresponding group at 57 weeks.

2-3-2. Tibialis anterior muscle weight

The muscle weights of the normobaric groups at 36 and 57 weeks were greater than that at 7 weeks (Fig. 3B). These results were similar in the hyperbaric groups. The muscle weight of the normobaric group at 90 weeks was lower than that at 57 weeks.

There were no differences in muscle weight between the normobaric and age-matched hyperbaric groups, irrespective of the age.

2-3-3. SDH activity of the tibialis anterior muscle

An age-related decrease in SDH activity was observed in the normobaric groups; the SDH activities of the muscle at 57 and 90 weeks were lower than that at 36 weeks and those at 7 and 36 weeks, respectively (Fig. 4). There were no differences in SDH activity of the muscle among the hyperbaric groups, irrespective of the age.

The SDH activity of the muscle in the hyperbaric group at 57 and 90 weeks was greater than that in the age-matched normobaric group.

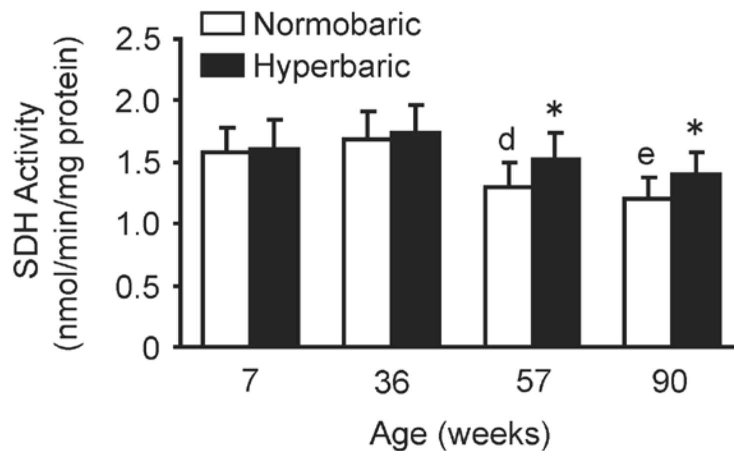

Fig. 4. Succinate dehydrogenase activities of the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from 6 animals. The mice in the hyperbaric group were exposed to 1.25 ATA with 36% oxygen for 6 h per day for 2 weeks. SDH, succinate dehydrogenase. $^{d}p < 0.05$ compared with the corresponding group at 36 weeks; $^{e}p < 0.05$ compared with the corresponding groups at 7 and 36 weeks; $^{*}p < 0.05$ compared with the age-matched normobaric group.

2-3-4. Fiber cross-sectional area in the tibialis anterior muscle

There were no differences in cross-sectional area of type IIA fibers among the normobaric groups, irrespective of the age (Fig. 5A). These results were similar in the hyperbaric groups.

There were no differences in cross-sectional area of type IIA fibers between the normobaric and age-matched hyperbaric groups, irrespective of the age.

The cross-sectional areas of type IIB fibers in the normobaric groups at 36 and 57 weeks were greater than those at 7 and 90 weeks (Fig. 5B). These results were similar in the hyperbaric groups.

There were no differences in cross-sectional area of type IIB fibers between the normobaric and age-matched hyperbaric groups, irrespective of the age.

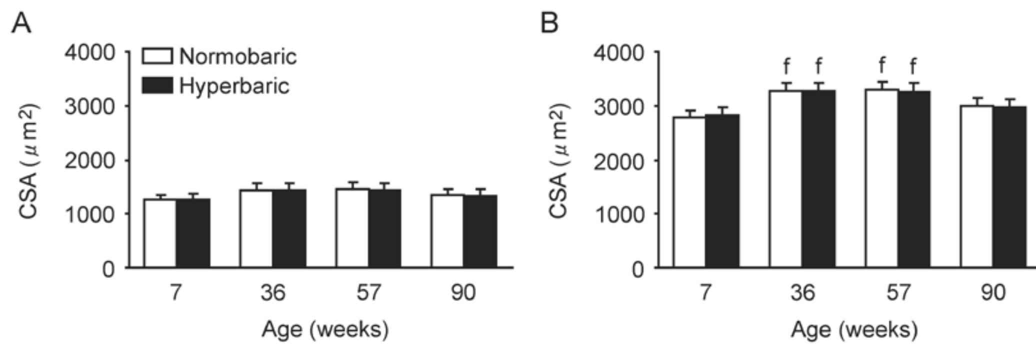

Fig. 5. Cross-sectional areas (CSAs) of type IIA (A) and type IIB (B) fibers in the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from 6 animals. The mice in the hyperbaric group were exposed to 1.25 ATA with 36% oxygen for 6 h per day for 2 weeks. CSA, cross-sectional area. $^{f}p < 0.05$ compared with the corresponding groups at 7 and 90 weeks.

2-3-5. Fiber SDH activity in the tibialis anterior muscle

The SDH activity of type IIA fibers in the normobaric group at 57 weeks was lower than that at 7 weeks (Fig. 6A). The SDH activity of type IIA fibers in the normobaric group at 90 weeks was lower than those at 7 and 36 weeks. The SDH activity of type IIA fibers in the hyperbaric group at 90 weeks was lower than that at 7 weeks. The SDH activity of type IIA fibers in the hyperbaric group at 90 weeks was greater than that in the age-matched normobaric group.

The SDH activity of type IIB fibers in the normobaric groups at 57 and 90 weeks was lower than that at 7 weeks (Fig. 6B). The SDH activity of type IIB fibers in the hyperbaric group at 90 weeks was lower than that at 7 weeks. The SDH activity of type IIB fibers in the hyperbaric group at 57 and 90 weeks was greater than that of the age-matched hyperbaric group.

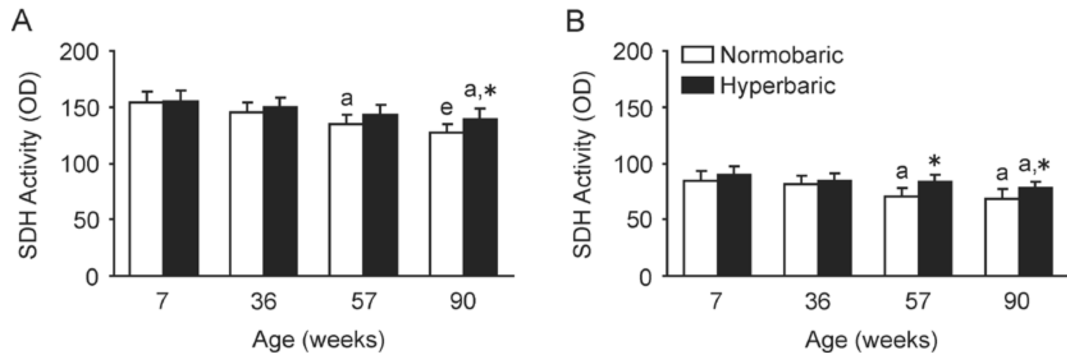

Fig. 6. Succinate dehydrogenase activities of type IIA (A) and type IIB (B) fibers in the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from 6 animals. The mice in the hyperbaric group were exposed to 1.25 ATA with 36% oxygen for 6 h per day for 2 weeks. SDH, succinate dehydrogenase; OD, optical density. $^{a}p < 0.05$ compared with the corresponding group at 7 weeks; $^{e}p < 0.05$ compared with the corresponding groups at 7 and 36 weeks; $^{*}p < 0.05$ compared with the age-matched normobaric group.

2-4. Discussion

An elevation in atmospheric pressure accompanied by high oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma [49, 93]. An increase in both atmospheric pressure and oxygen concentration enhances the mitochondrial oxidative enzyme activity and consequently increases oxidative metabolism in cells and tissues. Furthermore, an increase in atmospheric pressure and oxygen concentration increases carbon dioxide concentration, which in turn facilitates the release of oxygen from hemoglobin and causes the dilation of blood vessels. A hyperbaric chamber has been designed for performing the animal experiments [38]; the chamber consisted of an oxygen tank containing an oxygen concentrator and an air compressor, which automatically maintains the elevated

atmospheric pressure and oxygen concentration using a computer-assisted system. The optimal atmospheric pressure (1.25 ATA) and oxygen concentration (36%) required for obtaining effective responses was determined with regard to oxidative capacity in the neuromuscular system [38].

A previous study [55] demonstrated that young rats exposed to 1.25 ATA with 36% oxygen exhibited greater voluntary running activities than those maintained under normobaric conditions. It is also observed that oxidative enzyme activities of fibers in the soleus and plantaris muscles and of spinal motoneurons innervating these muscles increased following exposure to mild hyperbaric oxygen [55]. These findings suggest that the adaptation of neuromuscular units to exposure to mild hyperbaric oxygen enhances the oxidative capacity in muscle fibers and motoneurons, which promotes the function of the neuromuscular units. Furthermore, previous studies [61, 107] revealed that exposure to 36% oxygen at 1.25 ATA inhibited the growth-related increase in blood glucose levels of type 2 diabetic rats and in blood pressure levels of spontaneously hypertensive rats. Exposure to mild hyperbaric oxygen inhibited both the growth-related transition of fiber types from high- to low-oxidative and the decrease in oxidative enzyme activity of fibers in the soleus and plantaris muscles of type 2 diabetic rats [54, 105]. It is suggested that exposure to mild hyperbaric oxygen reduces the age-related decrease in the oxidative capacity of skeletal muscles, because exposure to mild hyperbaric oxygen facilitates the turn-over of oxidative metabolism, particularly of pathways in the mitochondrial TCA cycle.

Exercise is believed to be effective in maintaining and improving oxidative metabolism in cells and tissues. A previous study [37] observed that exercise is effective for the prevention of a decrease in the oxidative enzyme activity of type I and type II fibers in the soleus muscles of rats, which was induced by unloading. Furthermore, a previous study [106] found that running exercises served to inhibit the growth-related transition of fiber types from high- to low-oxidative in the soleus muscle of rats with type 2 diabetes, although this inhibition was observed only in rats that ran more than 7 km per day.

Atrophy, loss, and decreased oxidative enzyme activity of fibers in skeletal muscles have been observed with age [35, 39]. Muscle atrophy in old rats is associated with a decrease in activity levels of certain enzymes involved in oxidative metabolism [7].

These changes in skeletal muscles of rats in the initial stages of aging (60–65 weeks) are considered to be due to the age-related disuse of skeletal muscles, which results in the lowering of oxidative capacity of individual fibers. A previous study [2] observed that 96-week-old rats retained the capacity to increase the oxidative enzyme activity and mitochondrial density of skeletal muscles in response to endurance exercises. Furthermore, a previous study [40] observed that voluntary running exercises prevented atrophy of type II fibers as well as the decrease in oxidative enzyme activity of type I and type II fibers in rats in the initial stages of aging (65 weeks). Therefore, it is expected that a reduction in the decrease of oxidative metabolism in skeletal muscles, which was induced by exposure to mild hyperbaric oxygen as well as by aerobic exercise, should treat fiber atrophy and the decrease in oxidative capacity of skeletal muscles during the initial stages of the aging process.

The fibers of the tibialis anterior muscle in mice were classified into 2 types on the basis of staining intensities for the ATPase activity: type IIA and type IIB. In normobaric mice, type IIA fibers were smaller than type IIB fibers, irrespective of the age (Fig. 5). Type IIA fibers are more effective in supplying oxygen and nutrients for oxidative metabolism from capillaries, which are located close to the membrane, because of their small sizes. These indicate that type IIA fibers can work at a relatively low intensity and have more prolonged activity than do type IIB fibers. In this study, a reduction in cross-sectional area of type IIB fibers (Fig. 5B), but not type IIA fibers (Fig. 5A), was observed at 90 weeks. Low-intensity and prolonged activities, which are performed presumably using type IIA fibers, continue during increasing age, while high-intensity and short activities, which are performed presumably using type IIB fibers, decrease with increasing age. These indicate that type IIB fibers become less active with increasing age and, therefore, facilitate disuse-induced atrophy as observed in Fig. 5B. In this study, there were no differences in cross-sectional area of type IIA or type IIB fibers between the normobaric and age-related hyperbaric mice (Fig. 5). Therefore, exposure to mild hyperbaric oxygen had no effect on fiber cross-sectional area in the muscle. This view does not match previous expectations and is inconsistent with the findings observed in relation to exercise [40].

Exposure to mild hyperbaric oxygen reduced the age-related decrease in the oxidative enzyme activity of the tibialis anterior muscle (Fig. 4). Similarly, exposure to

mild hyperbaric oxygen reduced the oxidative enzyme activity of type IIB fibers in the muscle at 57 weeks (initial stage of aging) and those of type IIA and type IIB fibers at 90 weeks (middle to late stages of aging) (Fig. 6). The changes in the oxidative enzyme activity of the tibialis anterior muscle by exposure to mild hyperbaric oxygen corresponded well with that of muscle fibers. It is concluded that exposure to mild hyperbaric oxygen used in this study reduces the age-related decrease in the oxidative capacity of skeletal muscles because of the increased oxidative metabolism in cells and tissues.

Chapter 3
Exposure to Mild Hyperbaric Oxygen Activates Proliferation of Epidermal Basal Cells in Mouse Skin

3-1. Introduction

Epidermal basal cells are divided in the basal layers of the epidermis, differentiate into keratinocytes, and eventually desquamate from the skin surface [42, 85]. These regeneration processes of the skin, called turn-over, are important for maintaining the beauty and health of skin. However, the proliferative activity of epidermal basal cells and the turn-over speed decrease with age [23, 84], due to which the skin undergoes dramatic age-related changes in its mechanical properties, including tissue hydration and transepidermal water loss [83, 100].

Oxygen, which is necessary for proliferation, is carried to epidermis via the blood flow in the microcirculation and is utilized during proliferation of epidermal basal cells. However, it has been reported that 40% of blood flow at the forehead of 60-year-old women decreased compared to that of 25-year-old women [59]. It has also been reported that blood flow and partial oxygen pressure decrease with age [52]. One of the reasons for decreased blood flow is that capillary vessels degenerate with age. Previous studies [8-10, 27] have shown that with increasing age, the structure of microcirculation in the skin changes and the number and density of capillary vessels decrease.

The delay in the turn-over speed with age may be due to the decrease in the oxygen supply to the epidermis and, therefore, it is hypothesized that adequate oxygen supply to the skin may accelerate the turn-over speed of aged skin by enhancing the proliferative activity of epidermal basal cells [102]. Hence, if the proliferation of epidermal basal cells is activated, the health and beauty of the skin could be maintained, regardless of the age.

Exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen induces an increase in the blood flow and the dissolved oxygen concentration in the plasma [25, 80]. It is previously reported that exposure to mild hyperbaric oxygen improves ultraviolet B (UVB)-induced skin pigmentation in humans [70]. This study examined whether exposure to mild hyperbaric oxygen is effective in accelerating the proliferative activity of epidermal basal cells in mouse skin. Exposure to mild hyperbaric oxygen is

used for increasing the oxidative metabolism of epidermal basal cells, and therefore, is expected to accelerate excretion of the pigment melanin in differentially aged (15, 34, and 55 weeks old) hairless mice.

3-2. Materials and methods

This study was approved by the Experiment Committee of Kao Corporation (Tokyo, Japan). All experimental procedures, including experimental animal care, were conducted in accordance with the Guidelines of the National Institute of Health for the Care and the Use of Laboratory Animals of the Physiological Society of Japan.

3-2-1. Experimental animal care and treatment

Five-, 34-, and 55-week-old female hairless mice (Hos: HR-1, Hoshino Laboratory Animals Inc., Ibaragi, Japan) were used in this study. All mice were fed for 1 wk before the experiment to adapt to new surroundings. Food and water were provided ad libitum to all mice.

In each age group, mice were divided into the controls (Control, n = 7), 1 wk of exposure to mild hyperbaric oxygen (1-wk MHO, n = 7), and 2 wks of exposure to mild hyperbaric oxygen (2-wks MHO, n = 7). All mice were individually housed in same-sized cages in a room maintained under controlled 12 h light/dark cycles (lights switched off from 20:00 to 08:00) at a temperature of 22 ± 2°C with a relative humidity of 45%–55%.

The mice in the MHO groups were exposed to 1.25 ATA with an oxygen concentration of 36% automatically maintained by a computer-assisted system. The mild hyperbaric oxygen chamber is 180 cm long and has a diameter of 70 cm. The size of the chamber allows multiple mice (up to 20 cages) to be housed simultaneously. The mice in the MHO groups were exposed to the hyperbaric environment for 6 h (from 11:00 to 17:00) and were placed under normobaric conditions (1 ATA with 21% oxygen) for 18 h (from 17:00 to 11:00). The mice in the Control groups were placed under normobaric conditions for 24 h.

3-2-2. Blood sampling for oxidative stress test

Blood samples were obtained from the tail of fully conscious mice and evaluated

photometrically. A free radical and antioxidant potential determining device (Free Radical Analytical System 4; Health & Diagnostics, Grosseto, Italy) was used to automatically measure the level of derivatives of reactive oxygen metabolites (dROMs). The level of dROMs was used as an index to determine the level of oxidative stress (oxidant capacity) by measuring the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize *N,N*-diethyl-*p*-phenylenediamine. The level of dROMs was expressed in CARR units (1 U CARR corresponds to 0.08 mg hydro-peroxidase/100 mL H_2O_2).

3-2-3. Skin sampling and analysis

Following blood sampling, mice were sacrificed by overdose of sodium pentobarbital. Skin samples were isolated from the back skin of mice. Skin sample (1 × 1 cm) was fixed in 10% formalin solution for making serial skin sections. The formalin-fixed skin samples were embedded in paraffin and cut at 5-µm thickness. The sections were stained with hematoxylin and eosin (HE) solution. The preparations of HE-stained sections were sent to Sapporo General Pathology Laboratory Inc. (Sapporo, Japan) for analyses. Epidermal thickness was measured at 20 points in 3 different parts of each sample, which excluded hair follicles and excretory ducts of sweat glands, and the average value was calculated. The number of epidermal basal cells in 5 mm of epidermis, which excluded hair follicles and excretory ducts of sweat glands, was counted at 3 different points and the average value was calculated.

The proliferative activity of epidermal basal cells was obtained by calculating the percentage of epidermal basal cells exposed to mild hyperbaric oxygen per epidermal basal cells normobarically exposed, by using the following calculation formula: proliferative of epidermal basal cells (%) = (number of epidermal basal cells in the MHO group/number of epidermal basal cells in the age-matched Control group) × 100.

3-2-4. Statistical analysis

The data were expressed as mean ± standard deviation. Univariate analysis was used to evaluate differences among the Control, 1-wk MHO, and 2-wks MHO groups. When the differences were found to be significant, further comparisons were made by performing post hoc tests. A probability level of 0.05 and 0.01 was considered to be

statistically significant.

3-3. Results

There were no differences in the body weight (Fig. 7), redacted relative iron ion concentrations (Fig. 8a), or the level of dROMs (Fig. 8b) among the Control, 1-wk MHO, and 2-wks MHO groups, irrespective of the age.

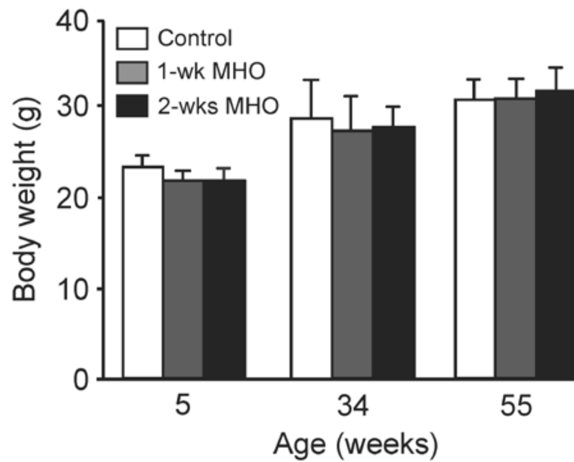

Fig. 7. Body weights of mice at different ages. The data are indicated as mean and standard deviation obtained from 7 mice. MHO, mild hyperbaric oxygen.

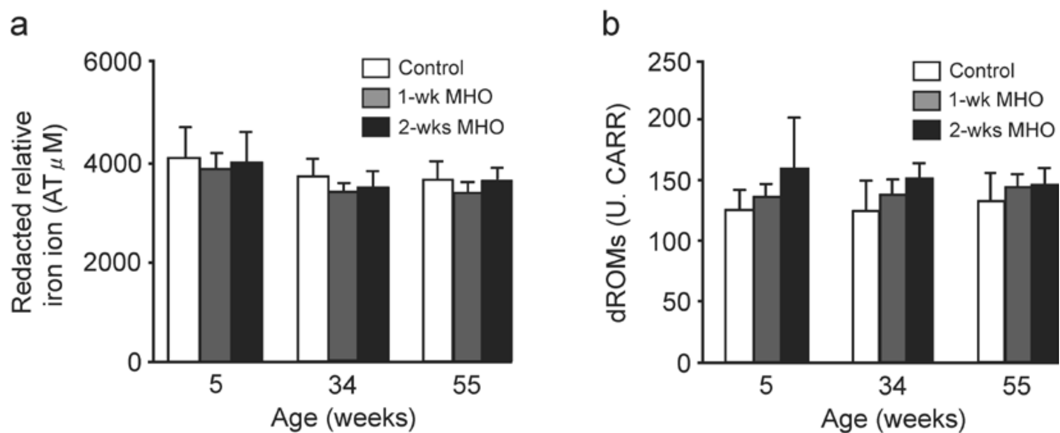

Fig. 8. Redacted relative iron ion (a) and dROMs (b) in mice of different ages. The data are indicated as mean and standard deviation obtained from 7 mice. dROMs, derivatives of reactive oxygen metabolites; MHO, mild hyperbaric oxygen.

Fig. 9 shows the photos of the hematoxylin and eosin-stained micrographs of the

epidermis. No unusual histological features or infiltration of inflammatory cells was observed in any of the epidermal samples obtained from all groups.

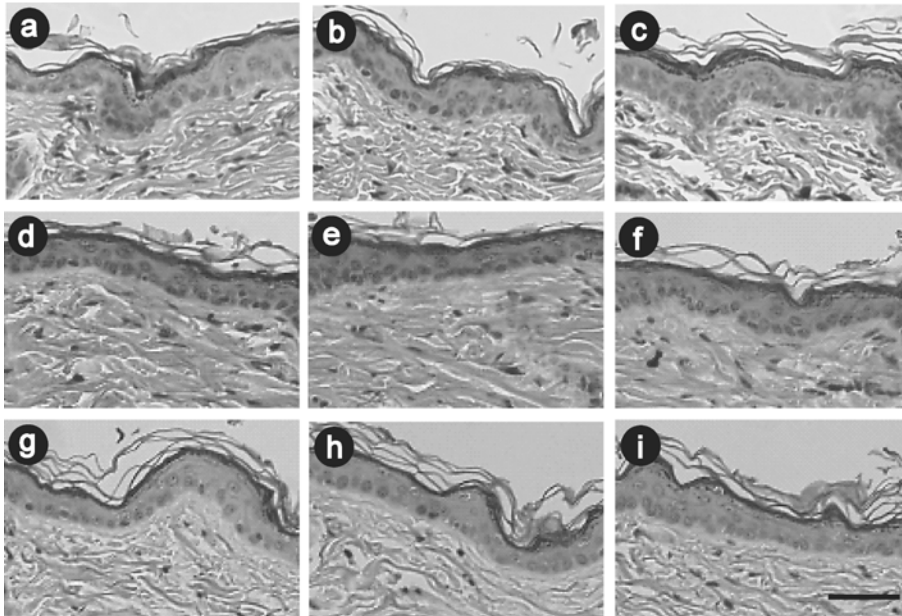

Fig. 9. Hematoxylin and eosin staining of skin sections from Control mice (a, d, and g) and 1-wk (b, e, and h) and 2-wks (c, f, and i) MHO mice. Scale bar = 50 μm.

There were no differences in the epidermal thickness among the Control, 1-wk MHO, and 2-wks MHO groups, irrespective of the age (Fig. 10).

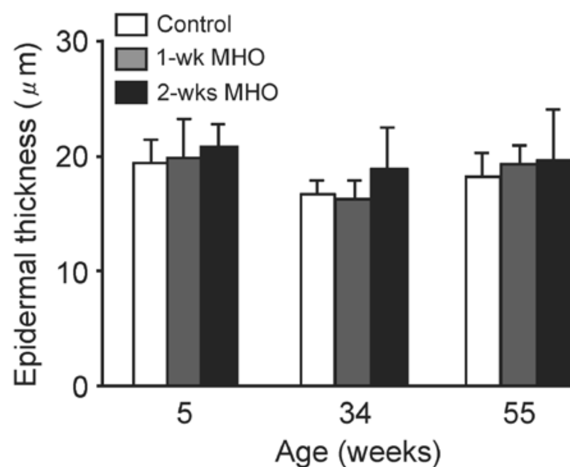

Fig. 10. Epidermal thickness in mice of different ages. The data are indicated as mean and standard deviation obtained from 7 mice. MHO, mild hyperbaric oxygen.

The number of epidermal basal cells was greater in the 1-wk MHO group than in the Control group, irrespective of the age (Fig. 11). In 5-wk-old mice, the number of epidermal basal cells was the highest in the 2-wk MHO group, greater than that in the Control and 1-wk MHO groups. In mice aged 34 and 55 wks, the number of epidermal basal cells in the 2-wk MHO group was greater than that in the Control group.

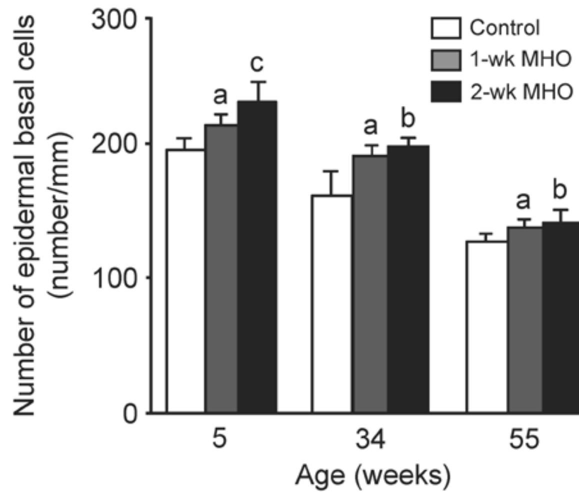

Fig. 11. Numbers of epidermal basal cells in mice of different ages. The data are indicated as mean and standard deviation obtained from 7 mice. MHO, mild hyperbaric oxygen. $^{a}p < 0.05$ compared with Control; $^{b}p < 0.01$ compared with Control; $^{c}p < 0.01$ compared with Control and $p < 0.05$ compared with 1-wk MHO.

The proliferation of epidermal basal cells was greater in the 1-wk MHO group than in the Control group, irrespective of the age (Fig. 12). In mice aged 5 wks, the proliferation of epidermal basal cells in the 2-wk MHO group was greater than that in both the Control and 1-wk MHO groups. In mice aged 34 and 55 wks, the proliferation of epidermal basal cells in the 2-wk MHO group was greater than that in the Control group.

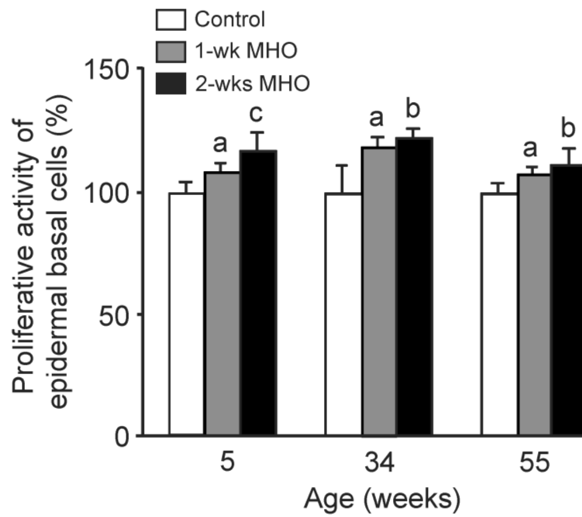

Fig. 12. Proliferative activities of epidermal basal cells in mice of different ages. The data are indicated as mean and standard deviation obtained from 7 mice. MHO, mild hyperbaric oxygen. [a]$p < 0.05$ compared with Control; [b]$p < 0.01$ compared with Control; [c]$p < 0.01$ compared with Control and $p < 0.05$ compared with 1-wk MHO.

3-4. Discussion

The epidermis receives oxygen from the atmosphere directly and, therefore, the epidermis can sustain higher oxygen levels than many internal tissues [69]. Previous studies [31, 101] have reported that oxygen concentration up to 20% increases the proliferation of human keratinocytes. Furthermore, cultures of the human epidermis that were incubated in 20% oxygen ambience attained greater thickness than those at 2% oxygen [32, 69]. In addition, wounds covered by oxygen impermeable plastic films take a longer time to recover from injury than wounds healing under normal conditions [47]. These results suggest that under in vivo conditions also, adequate oxygen supply to the epidermis accelerates the proliferation of epidermal basal cells.

Previous studies [46, 97] have reported that exposure to hyperbaric oxygen induces an increase in the amount of dissolved oxygen in the plasma. Exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen has been widely used to increase the oxidative metabolism and enhances the oxidative enzyme activity of cells and tissues [32, 38, 55]. Under the mild hyperbaric conditions used in this study, oxygen concentration was 1.72 times higher and atmospheric pressure was 1.25 times greater. It is estimated that the dissolved oxygen concentration into body fluids was approximately

2.3 times greater than that under normal conditions. Therefore, it is plausible that the increased dissolved oxygen concentration increases oxidative metabolism.

It is well known that a high concentration of oxygen causes unusual changes such as overproduction of reactive oxygen species [60] and/or increasing invasive inflammatory cells [16]. In this study, no increase in oxidative stress (Fig. 8b) or invasive cells (Fig. 9) in the epidermal organization of any group was observed. These data indicate that exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen is not harmful for the mouse skin.

The number of epidermal basal cells decreases with age; the number of epidermal basal cells in the Control group of mice aged 55 wks was about 60% of that in mice aged 5 wks (Fig. 11). After exposure to mild hyperbaric oxygen for 2 weeks (the 2-wks MHO group), the number of epidermal basal cells in 34-wk-old mice was similar to that observed in the Control group of 5-wk-old mice. This result suggests that exposure to mild hyperbaric oxygen improves age-related epidermal organization. However, after exposure to mild hyperbaric oxygen (the 2-wks MHO group) of 55-wk-old mice, the number of epidermal basal cells did not reach the levels of those in the Control groups of mice aged 5 wks and 34 wks. These results suggest that the proliferative activity of epidermal basal cells was improved by exposure to mild hyperbaric oxygen; however, the efficacy of exposure to mild hyperbaric oxygen was lower in older mice.

The proliferation of epidermal basal cells was significantly increased in the MHO groups, irrespective of the age. It is verified that increased oxygen concentration by exposure to mild hyperbaric oxygen induced proliferation of keratinocytes in vivo similar to that in vitro. In particular, mice in the MHO groups aged 34 wks showed the highest proliferative activity of epidermal basal cells after exposure to mild hyperbaric oxygen (Fig. 12). These results suggest that the difference in the proliferative activity of epidermal basal cells with respect to the responsiveness to increased oxygen concentration induced by exposure to mild hyperbaric oxygen is almost not age-related.

A previous study [26] showed that the rapidity of proliferation of epidermal basal cells in cases of psoriasis or acanthosis [26] is faster than that of healthy skin. In this study, however, no change in the thickness of the epidermis was observed after exposure to mild hyperbaric oxygen. Combined with a previous study [70], these data suggest that exposure to mild hyperbaric oxygen accelerates not only proliferation of

epidermis but also its turn-over speed. Further studies are needed to evaluate the RNA expression level of keratin fibers, transglutaminase, and cornified envelope proteins.

In conclusion, exposure to mild hyperbaric oxygen used in this study accelerated the proliferative activity of epidermal basal cells in the mouse skin via increased oxidative metabolism.

Chapter 4
Exposure to Mild Hyperbaric Oxygen Improves UVB Irradiation-induced Melanin Pigmentation and Diminishes Senile Spot Size

4-1. Introduction

Cells and tissues use oxygen as a central role in oxidative phosphorylation and produce energy for maintaining their metabolism and function. Epidermis has direct access to oxygen and obtains much oxygen from the atmosphere; therefore epidermis can maintain higher oxygen level than many internal tissues [69]. When ultraviolet (UV) is irradiated on skin, melanocytes are assumed to exhibit a series of oxidative stress [86], suggesting that much oxygen is needed for disappearance of oxidative stress and for repair of damaged epidermis.

Cells and tissues receive oxygen from two ways; one is from oxygen binding to hemoglobin in red blood cells, which flow in blood vessel, and another is from oxygen dissolved in the tissue fluids including the plasma. Previous studies [25, 80] observed that exposure to hyperbaric oxygen induces an increase in the amount of dissolved oxygen in the plasma. Hyperbaric exposure at 2-3 ATA with 100% oxygen is generally used for therapy of temporary hypoxia [46], tissue repair after burn [97], intractable ulcer [64, 91], and open fractures and crush injuries [4].

Exposure to mild hyperbaric oxygen at moderate atmospheric pressure with high oxygen concentration has been investigated [38]; hyperbaric exposure at 1.25 ATA with 36% oxygen enhances the oxidative enzyme activity of cells and tissues [38, 55], therefore inducing inhibition in the growth-associated increase in blood glucose level of type 2 diabetic Goto-Kakizaki rats [24, 105, 107] and in blood pressure level of spontaneously hypertensive rats [61]. These studies [24, 61, 105, 107] concluded that exposure to mild hyperbaric oxygen at moderate atmospheric pressure with high oxygen concentration has a beneficial effect on oxidative metabolism in cells and tissues although the amount of dissolved oxygen is greater by hyperbaric exposure at 2-3 ATA with 100% oxygen than by that at 1.25 ATA with 36% oxygen.

It is suggested that keratinocyte proliferation and epidermal cell regeneration are activated by an enhanced oxidative metabolism, which is induced by exposure to mild

hyperbaric oxygen. This study examined whether hyperbaric exposure at 1.25 ATA with 32% oxygen has an effect on UVB irradiation-induced melanin pigmentations of upper arm skins and on senile spot sizes of faces.

4-2. Materials and methods

This study was approved by the Experiment Committee of Kao Corporation, Tokyo, Japan and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and the Guiding Principles published by the Physiological Society of Japan. All subjects participated in this study agreed with informed consent prepared by Kao Corporation.

4-2-1. The 1st experiment

Erythema and the subsequent melanin pigmentation of 14 healthy male subjects (31-44 years old) were induced by a UVB irradiation device (FL20SE UV light; Toshiba, Japan) [92]. None of these subjects had skin diseases or history of photosensitivity. In order to determine the minimal exposure dose (MED) for recognizing erythema of individual subjects, UVB with a peak radiation wavelength of 350 nm was irradiated from 190.04 to 427.59 mJ/cm^2 with increment of 25% on 1.5 cm^2 in diameter at 6 different sites of the ventral side of their right upper arms. The MED was assessed 24 h after UVB irradiation. Thereafter, the UVB, which corresponded to the double dose of individual MEDs, was irradiated on 1.5cm^2 in diameter of the ventral side of their left upper arms.

In this study, erythema was observed after 4 weeks of UVB irradiation. Therefore, the subjects were assigned into the control (CO; n = 7) and mild hyperbaric oxygen (HO; n = 7) groups as these 2 groups had the similar degree of erythema. Subjects in the HO group were exposed to 1.25 ATA with 32% oxygen for 1 h per day, 3 times per week during 4 weeks. The mild hyperbaric chamber was designed to automatically maintain an increased level of oxygen concentration and atmospheric pressure. Subjects in the CO group were maintained under a normobaric environment at 1 ATA with 21% oxygen.

The luminance value (L*), which is used as an index for the level of melanin pigmentation, was determined by an image-processing method using a videomicroscope

interfaced with a computer 1, 2, 3, and 4 weeks after exposure to mild hyperbaric oxygen. The L* is one of the three indices of chromatically designated L*a*b*, which is matching to the Commission International de l'Eclairage standard observer response [98]. The change in L* of spot (ΔL*pig.), which was irradiated by UVB, was measured every week by a colormeter (CM2600D; Minolta, Tokyo, Japan). In order to cancel the change in skin color during test period, we measured the change in skin color on control spot (ΔL*skin) next to the spot, which was irradiated by UVB. The ΔΔL* value (ΔΔL* = ΔL*pig. - ΔL*skin) was determined to assess the change in melanin pigmentation on the spot.

4-2-2. The 2nd experiment

Seven healthy female subjects (30-41 years old), who had senile spots on their faces, were exposed to 1.25 ATA with 32% oxygen for 1 h per day, 2 times per week during 12 weeks. None of these subjects had skin diseases or history of photosensitivity.

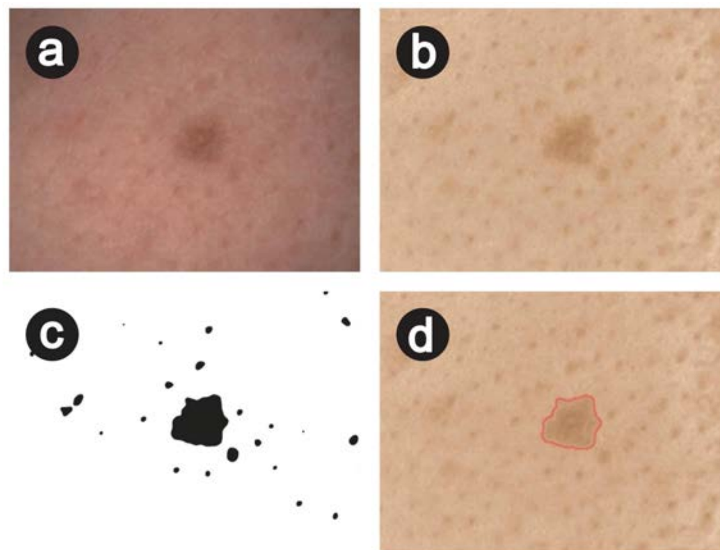

Fig. 13. Image processing for the senile spot size measurement. The original image (a) was modified to the one including noise-reduced component (b). The image was converted to the binaly picture (c) for making clear contour of the spot, and thereafter, its size was measured by an image-processing soft (d).

Total 13 senile spots (1 or 2 spots per subject) were selected. The images of senile spots were taken by a digital microscope (Hi-Scope Advanced KH-3000; HiROX,

Tokyo, Japan) before exposure to mild hyperbaric oxygen as baseline and after 3, 7, and 12 weeks of exposure to mild hyperbaric oxygen (Fig. 13). The images were separated into melanin and hemoglobin chromophores [74]. The images of melanin chromophores were converted to binaly pictures for making clear contours of spots, and thereafter, their sizes were measured by an image-processing soft (Photoshop CS4; Adobe Systems, Tokyo, Japan).

4-2-3. Statistics

The data were presented as mean and standard deviation. One-way analysis of variance (ANOVA) was used to evaluate the time-course changes in melatonin pigmentation and senile spot size. When there were overall significant differences based on the ANOVA analyses, individual group comparisons were made using Scheffé's post hoc tests. A probability level of 0.05 was considered to be statistically significant.

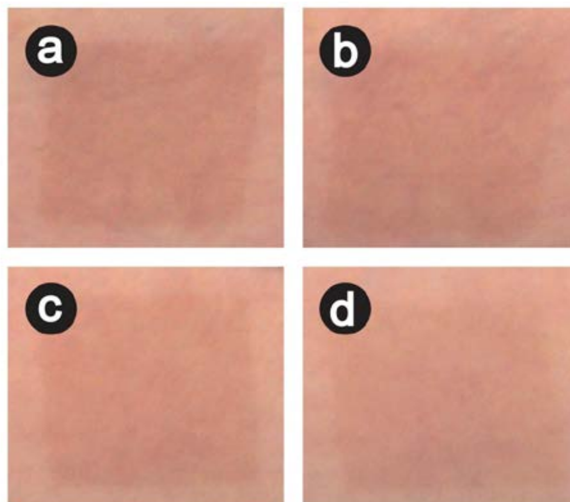

Fig. 14. Photographs of ultraviolet B-induced pigmentations in the control (a and b) and mild hyperbaric oxygen (c and d) groups. a and c, control and hyperbaric groups before exposure to mild hyperbaric oxygen, respectively; b and d, control and mild hyperbaric groups after 4 weeks of exposure to mild hyperbaric oxygen, respectively.

4-3. Results

Fig. 14 shows photographs of melanin pigmentations in the CO and HO groups before and 4 weeks after exposure to mild hyperbaric oxygen. The melanin pigmentations turned light following exposure to mild hyperbaric oxygen; the ΔΔL* of

the HO group was significantly higher than that of the CO group after 4 weeks of exposure to mild hyperbaric oxygen although there was no difference in the ΔΔL* between the CO and age-matched HO groups during 1 to 3 weeks of exposure to mild hyperbaric oxygen (Fig. 15).

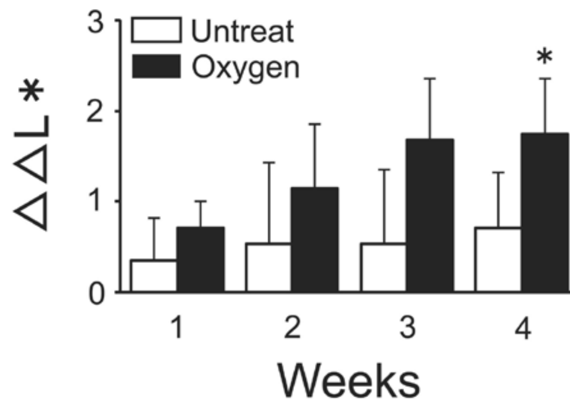

Fig. 15. Changes in pigmentation (ΔΔL*) following exposure to mild hyperbaric oxygen. The data are indicated as mean and standard deviation obtained from 7 subjects. Subjects in the HO group started exposure to mild hyperbaric oxygen after 4 weeks of recovery from ultraviolet-B irradiation because erythema was observed at 4 weeks after ultraviolet-B irradiation. *$p < 0.05$ compared with the age-matched CO group.

Fig. 16 shows photographs and images of senile spots before and 12 weeks after exposure to mild hyperbaric oxygen. Senile spot sizes gradually decreased following exposure to mild hyperbaric oxygen. The spot size after 12 weeks of exposure to mild hyperbaric oxygen was significantly smaller than that before exposure to mild hyperbaric oxygen; the average senile spot size after 12 weeks of exposure to mild hyperbaric oxygen was 82% compared with that before exposure to mild hyperbaric oxygen (Fig. 17).

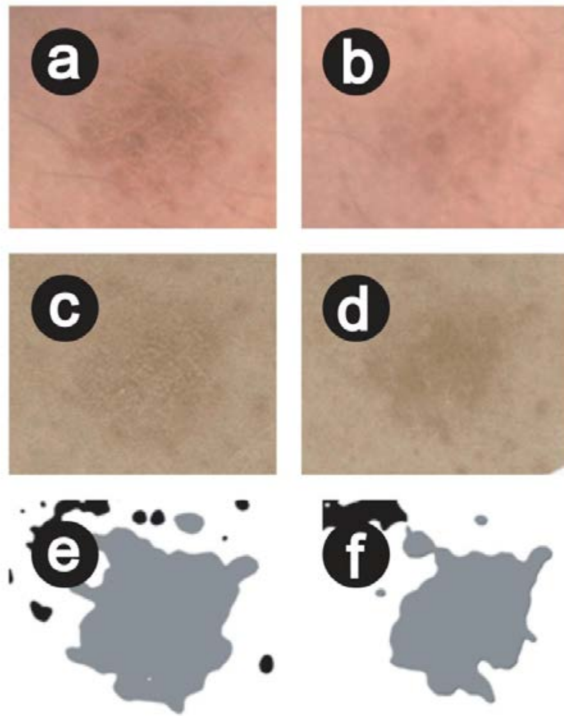

Fig. 16. Images of the senile spots before (a, c, and e) and 12 weeks after exposure to mild hyperbaric oxygen (b, d, and f). a and b, cross-polarized images; c and d, images including components of melanin pigmentation; e and f, binary pictures.

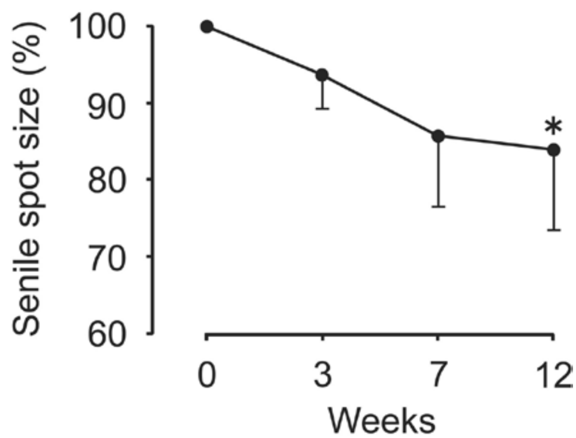

Fig. 17. Changes in senile spot size (%) following exposure to mild hyperbaric oxygen. The data are indicated as mean and standard deviation obtained from 13 spots of 11 subjects. $*p < 0.05$ compared with the value before exposure to mild hyperbaric oxygen.

4-4. Discussion

Hyperbaric exposure at a pressure greater than 1 ATA, usually at 2-3 ATA, with

100% oxygen has been successfully used as an adjunctive therapy for many clinical disorders related both to ischemia and/or hypoxia [4, 46, 64, 91, 97]. In contrast, a previous study [19] reported that cataracts, particularly nuclear cataracts which are major cause of loss of lens transparency, in 17- to 18-month-old guinea pigs was induced by hyperbaric exposure at 2.5 ATA with100% oxygen for 2-2.5 h, 3 times per week for up to 100 times. Similarly, myopia and cataract developed in human lenses by prolonged hyperbaric exposure at 2-2.5 ATA with 100% oxygen for 1.5 h, 1 time per day from 150 to 850 times [78], but have been observed rarely after only 48 times [18].

Exposure to hyperbaric oxygen is considered to cause excessive production of reactive oxygen species (ROS) in several tissues and organs [68, 75-77] and ROS have the ability to act as important signaling molecules [90]. A standard procedure of hyperbaric exposure at 2-3 ATA with 100% oxygen has the potential to induce and accelerate myopia and cataract. Furthermore, hyperbaric oxygen-induced oxidative stress levels depend not only on the pressure but also on the exposure duration; the pressure from 2.5 to 3 ATA and the duration from 90 to 120 min resulted in a pronounced change in oxidative stress level [75, 76].

Formation of ROS [108] and lipid peroxides [71] in rat brain increases after exposure to hyperbaric oxygen at more increased pressure level. Furthermore, exposure to hyperbaric oxygen causes elevation of blood pressure level and lowering of both heart rate and blood glucose level, which were augmented in the presence of hypertension, diabetes, and both hypertension and diabetes [1]. Especially, diabetic patients cause a greater elevation in blood pressure level after exposure to hyperbaric oxygen compared to hypertensive patients and healthy individuals.

In this study, exposure to mild hyperbaric oxygen at 1.25 with 32% oxygen was used because these moderate pressure and oxygen concentration enhance the oxidative enzyme activity of cells and tissues [38, 55]. Previous studies observed that exposure to mild hyperbaric oxygen at 1.25 ATA with 36% oxygen induced an inhibition in the growth-associated increase in blood glucose level of type 2 diabetic Goto-Kakizaki rats [24, 105, 107] and in blood pressure level of spontaneously hypertensive rats [61]. Furthermore, overproduction of ROS in spontaneously hypertensive rats was inhibited after exposure to mild hyperbaric oxygen [61]. These studies [24, 61, 105, 107] conclude that exposure to mild hyperbaric exposure at moderate atmospheric pressure

with high oxygen concentration has a beneficial effect on enhancement and improvement in oxidative metabolism of cells and tissues.

Many agents, in which are mostly ointments, have been investigated for improvement in damaged skin by erythema, peeling, and stinging [72, 99, 104]. Erythema and the subsequent pigmentation were suppressed and in a parallel manner by corticosteroids and indomethacin which were applied immediately after UVB irradiation [92]. Chemical mediators, especially prostaglandins, released in the inflammatory process have an important role in the induction of erythema [3, 14]. Histamine is also involved in the erythema formation in skins of animals and humans [21], probability by enhancing prostaglandins synthesis in UVB-irradiated skin in the earlier phase of inflammation [79]. Furthermore, these mediators stimulate melanocytes of skins in animals [33, 73] and humans [94]. It is suggested that the suppression of UVB irradiation-induced pigmentation is due at least to the reduction in prostaglasndins synthesis through the inhibition of cyclooxygenase by indomethacin and the induction of annexin of lipocortin by corticosteroids [15].

Unlike many internal tissues, epidermis has direct access to atmospheric oxygen and obtains much oxygen from the atmosphere; therefore, epidermis can maintain higher oxygen levels than internal tissues [69]. However, chronic wounds of the skin in regions of poor blood supply, e.g., chronic ulcers, have been proposed to be refractory to healing owing to insufficient blood supply and oxygen availability. Therefore, patients utilize dissolved oxygen increased by exposure to hyperbaric oxygen or the wound is treated topically with elevated oxygen concentrations. Furthermore, melanocytes are assumed to exhibit a series of oxidative stress during UVB irradiation-induced melanogenesis [86], suggesting that much oxygen is needed for repair and maintenance of epidermis after erythema formation. These views mean that epidermis is exposed to local tissue hypoxia, which was induced by limited supply of oxygen under conditions of chronic wound and melanogenesis.

It is postulated that increased oxygen induced by exposure to mild hyperbaric oxygen is effective for repair from damage in epidermis, which was induced by UVB-irradiation. In this study, melanin pigmentation lightened after 4 weeks of exposure to mild hyperbaric oxygen (Fig. 14). It is suggested that the increase in blood flow and dissolved oxygen into damaged epidermis was induced by exposure to mild

hyperbaric oxygen. Although inhibition in overproduction of ROS under epidermis formation would be expected following exposure to mild hyperbaric oxygen, the changes in ROS level was not examined in this study. Further studies including animal experiments are needed to elucidate whether exposure to mild hyperbaric oxygen has an effect on inhibition in overproduction of ROS, which may be linked to epidermis formation.

A previous study [74] observed seasonal changes in senile spot sizes of faces in 105 female subjects (20-60 years old). Total 115 senile spot sizes were determined quantitatively in 4 different seasons; March, June, September, and December. The senile spot sizes became largest in June, however, the change in senile spot size was a narrow variation within 5% range during a year. Therefore, it is concluded that there were no seasonal changes in senile spot size. In this study, the senile spot size after 12 weeks of exposure to mild hyperbaric oxygen was 82% compared with that before exposure to mild hyperbaric oxygen (Figs. 16 and 17). It is suggested that decreased spot sizes after exposure to mild hyperbaric oxygen are due to an increased oxidative metabolism, which changes were considered in our 1st experiment using UVB irradiation-induced melatonin pigmentation and its inhibition by exposure to mild hyperbaric oxygen.

Chapter 5
Conclusions

The mammals have 2 pathways for producing energy, so called anaerobic and aerobic pathways. Increased dissolved oxygen of tissue is one of the important factors which are subject to decide anaerobic or aerobic metabolism.

It is considered that one of the reasons of age-related change in aerobic metabolism [41] is caused by the decrease in oxygen supply to cells and tissues. This is because oxygen is supplied to body tissues by blood flow, but the volume of blood flow decrease with age [52]. Furthermore, arterial partial pressure of oxygen (P_aO_2) decreases with age, reflecting by the formula $P_aO_2 = 102 - (0.498 \times \text{age})$ [96]. Hence, increasing dissolved oxygen in aged body tissues by exposure to mild hyperbaric oxygen has a possibility of enhancing aerobic metabolism and leading to activation of body tissues.

In this study, exposure to mild hyperbaric oxygen at 1.25 with 32% oxygen was used because these moderate pressure and oxygen concentration enhance the oxidative enzyme activity of cells and tissues. Under conditions of mild hyperbaric oxygen at 1.25 ATA with 36% oxygen in these studies, the dissolved oxygen is estimated about 2.3 times greater compared to normal condition according to the Henry's law.

From the results of Chapter 2, the oxidative enzyme activity of the tibialis anterior muscle, which decreased with age, was suppressed at 57 and 90 weeks-old mice by exposure to mild hyperbaric oxygen. This result shows that exposure to mild hyperbaric oxygen can improve the decrease in aerobic metabolism of skeletal muscles at aged mice. It is reported that long-term aerobic excise appears to attenuate age-related reductions in muscle strength [43], therefore exposure to mild hyperbaric oxygen is considered to be useful for maintaining health of elderly adults as well as aerobic excise.

In Chapter 3, the effectiveness of exposure to mild hyperbaric oxygen for the proliferation activity of keratinocyte was examined in old mice at different weeks. The number of epidermal basal cells in 55-week-old mice was about 60% of that in 5-week-old mice. However, after exposure to mild hyperbaric oxygen for 2 weeks, the number of epidermal basal cells in 34-week-old mice recovered to the similar level of that of 5-week-old mice without unusual historical feature of epidermis. Exposure to

mild hyperbaric oxygen accelerated the proliferative activity of epidermal basal cells in the mouse skin via increased oxidative metabolism.

From the results of Chapter 3, it is considered that exposure to mild hyperbaric oxygen has a possibility to improve skin pigmentation which is caused with age. If the proliferation activity of keratinocyte is accelerated safely, it is considered that melanin pigmentation, which accumulates in stratum corneum, is discharged quickly by turn-over process of skin. To verify the efficacy of exposure to mild hyperbaric oxygen on human skin, the effects of mild hyperbaric oxygen on UVB irradiation-induced melanin pigmentation and on senile spot sizes of faces were examined in Chapter 4. Melanin pigmentation, which is artificially induced by UVB, lightened significantly after 4 weeks of exposure to mild hyperbaric oxygen. Moreover, senile spot sizes became small after 12 weeks of exposure to mild hyperbaric oxygen. It is concluded that exposure to mild hyperbaric oxygen used in this study accelerates both the fading in melanin pigmentation and the decrease in senile spot size.

These series of studies showed that exposure to mild hyperbaric oxygen is effective for the improvement in age-related changes of cells and tissues by increasing of aerobic metabolism. In addition, exposure to mild hyperbaric oxygen provides a possibility that it contributes to not only keep health but also maintain beauty of elderly adults.

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Original Articles

This dissertation is based on the following articles:

Nishizaka T, Nagatomo F, Fujino H, Nomura T, Sano T, Higuchi K, Takeda I, Ishihara A. Hyperbaric oxygen exposure reduces age-related decrease in oxidative capacity of the tibialis anterior muscle in mice. Enzyme Research, doi: 10.4061/2010/824763, 2010.

Nishizaka T, Nomura T, Sano T, Higuchi K, Nagatomo F, Ishihara A. Hyperbaric oxygen improves UVB irradiation-induced melanin pigmentation and diminishes senile spot size. Skin Research and Technology, 17: 332-338, 2011.