ABSTRACT: *Chlorella pyrenoidosa* (*C. pyrenoidosa*) has higher content of chlorophyll than any known other plants, and also has vitamins, minerals, dietary fiber and other substances. To identify the effect of *C. pyrenoidosa* on skin condition, we measured cutaneous arterial sympathetic nerve activity (CASNA), cutaneous blood flow and transepidermal water loss (TEWL) in rats in three separate trials. We further attempted to determine if *C. pyrenoidosa* was targeting the histamine H3 receptors by pretreating a separate group with thioperamide, a histamine H3 antagonist, and then observing the CASNA and cutaneous blood flow response to *C. pyrenoidosa* in a fourth trial. Intraduodenal administration of *C. pyrenoidosa* caused marked inhibition of CASNA as well as significant elevation of cutaneous blood flow in rats. TEWL, furthermore, significantly decreased on the dorsal skin of conscious hairless rats when their only source of fluids was *C. pyrenoidosa* suspended in water. However, when animals were pretreated with thioperamide, *C. pyrenoidosa* eliminated the response of CASNA and cutaneous blood flow. These findings suggest that *C. pyrenoidosa* reduces CASNA, increases cutaneous blood flow, and enhances the water-retaining ability of the skin, and that histamine H3 receptors may be involved in the CASNA and cutaneous blood flow response.

KEY WORDS: *Chlorella pyrenoidosa*, Cutaneous arterial sympathetic nerve activity, Cutaneous blood flow, Histamine, Transepidermal water loss

INTRODUCTION: *Chlorella pyrenoidosa* (*C. pyrenoidosa*) is a unicellular green alga that grows in fresh water. The principal components of chlorella that have been shown to have certain health benefits are chlorophyll, the organism’s cell walls, beta-carotene, and chlorella growth factor (Merchant et al., 2000). Chlorella growth factor contains amino acids, vitamins, proteins, nucleic acids and other substances (Merchant et al., 2000).

Good skin health is often evaluated using cutaneous arterial sympathetic nerve activity (CASNA), cutaneous blood flow (CBF) and transepidermal water loss (TEWL). We previously induced changes in CASNA that in turn affected CBF and TEWL through the intraduodenal (ID) administration of *Lactobacillus brevis* SBC8803 in separate trials conducted on rats (Horii et al., 2014). Furthermore, it was observed that dermal application of urea affected CASNA, CBF and TEWL in rats (Horii et al., 2011). Authors previously studied alterations in autonomic nerve activities and physiological phenomena in rats and could detect causal relationships between changes in autonomic nerve activities and physiological phenomena in rats and mice.

Since, a number of reports have shown that *C. pyrenoidosa* have many type of physiological effect such as stimulation of immune system, modulation of blood pressure and acceleration wound healing (Komiyama et al., 1986; Miyazawa et al., 1988; Merchant et al., 2001), we thus examined effects of ID administration of *C. pyrenoidosa* on CASNA, CBF and TEWL in rats, in order to identify actions of *C. pyrenoidosa* on the skin condition.

The regulation of important activities of the central nervous system such as neurotransmission and autonomic function implicates histamine. (Schwartz et al., 1991; Sakata et al., 1997). We focus on the response of CASNA and CBF may be realized via histaminergic neural function. In the histaminergic nervous system, the presynaptic H3-receptor mediates the autoinhibition of histamine release from histaminergic
neurons into synaptic clefts, and the affinity of the H3 receptor to histamine is much higher than that of the postsynaptic histaminergic H1 receptor (Arrang et al., 1983; Watanabe et al., 1984). Therefore, the sympathetic activation might occur via histaminergic H1-receptors, whereas sympathetic suppression might be mediated via histaminergic H3-receptors. In our previous study, intravenous (IV) pre-injection of thioperamide eliminated the CASNA and CBF response induced by dermal application of urea-containing cream (Horii et al., 2011).

We thus conducted a separate trial and administered a histamine H3 antagonist, thioperamide male salt, after the administration of C. pyrenoidosa and measured the CASNA and CBF response.

MATERIALS AND METHODS

Animals

Seven-week-old male Wistar rats (Kiwa Laboratory Animals, Co. Ltd., Wakayama, Japan), and male hairless Wistar Yagi rats (Japan SLC, Inc. Shizuoka, Japan) were used. The Wistar rats were used in the CASNA and CBF trials while the hairless rats were used in the TEWL trial. Rats were housed in a room maintained at 24±1°C with 12h light/dark cycles everyday. Food and water were available ad libitum. The animals were acclimated to the environment for at least 1 week before the experiment. The Institutional Animal Care and Use Committee of ANBAS Corporation approved all animal care and handling procedures.

Preparation of C. pyrenoidosa Powder Suspension and Thioperamide Maleate Solution

The C. pyrenoidosa powder was produced by Sun Chlorella Corp. (Kyoto, Japan, Chlorella pyrenoidosa Lot No. SCA2216). The powder was prepared by crushing the cell walls of using Dyno-Mill (WAB, Inc., Switzerland) and spray drying.

C. pyrenoidosa was suspended in distilled water (30 mg/250 ml) and a 1 ml solution was prepared for the CASNA and CBF trials. A 250-ml water bottle was filled with C. pyrenoidosa at the same concentration for use in the TEWL trial.

The solution of thioperamide maleate salt (2 mg/ml in physiological saline, Sigma-Aldrich Co., USA), a histamine H3 receptor antagonist, was prepared for the experiment using thioperamide.

Distilled water was used as a negative control in the CASNA, CBF and TEWL trials, while physiological saline was used as negative control in the CASNA and CBF trial using the histamine H3 antagonist thioperamide.

Determination of CASNA

Wistar male rats (C. pyrenoidosa group, n = 5; control group, n = 5) were used in the CASNA experiment. Food was removed 3 to 4 h prior to surgery on the day of the experiment. General preparation was performed as described previously (Horii et al., 2011).

For recording the CASNA, the sympathetic nerve that innervates the cutaneous artery was exposed through a longitudinal incision in the left femoral region. The distal end of the nerve was ligated and hooked up to a pair of silver wire electrodes for recording efferent nerve activity (Tanida et al., 2005a). Once the animal had stabilized, a baseline CASNA measurement was made 5 min prior to the ID administration of 1 ml of C. pyrenoidosa suspension or water. CASNA was then recorded for 60 min from the start of the ID administration. Specifically, electrical changes in CASNA were amplified and monitored by an oscilloscope. The raw data of the nerve activity were converted to standard pulses by a window discriminator, as described previously (Tanida et al., 2005a).

Determination of CBF in The Tail

Wistar male rats (C. pyrenoidosa group, n = 5; control group, n = 5) were also used in the CBF experiment. CBF was determined using a laser flow meter (ALF21, Advance Co., Tokyo) as described previously (Kobayashi et al., 2000). The animals were cannulated intraduodenally under urethane anesthesia (1 g/kg, intraperitoneal (IP) injection). The probe (tip diameter of 1 cm) of the laser flow meter was fixed to the proximal end of dorsal surface of the rat tail using surgical tape. Blood flow was measured 5 min prior to the administration of 1 ml of C. pyrenoidosa suspension or water and the resulting value was used as a baseline value. C. pyrenoidosa suspension or water was then ID administered and CBF data were collected for 60 min from the start of administration. CBF data were sampled with a Power-Lab analog-to-digital converter, and stored on a PC for off-line analysis.

Determination of TEWL

The hairless rats (C. pyrenoidosa group, n = 5; control group, n = 5) were used instead of the Wistar rats which were used in the previous trials, because measurement of TEWL requires a hairless surface. The hairless rats were therefore used so that TEWL could be measured on their hairless dorsal area.

The TEWL trial was conducted over a period of 4 days. Animals were housed individually over this 4-day period to prevent damage to the skin surface. The animals were anesthetized (pentobarbital anesthesia: 35 mg/kg of body weight, IP) to immobilize them at the time of measurement. TEWL was measured beginning at 14:00 during the light period. VapoMeter (Delfin Technologies Ltd., Kuopio, Finland) was used to measure TEWL as described previously (Horii et al., 2014).

Baseline measurements were taken on Day 0 on immobilized animals which had not been administered the C. pyrenoidosa suspension or distilled water. Once these baseline measurements were taken, the C. pyrenoidosa group was given ad-libitum access to C. pyrenoidosa in the drinking water and the control group to distilled water throughout the entire test period. TEWL values were obtained for all 3 subsequent test days for each animal and the mean of these values was used.
Effects of *C. pyrenoidosa* on skin health

Determination of The Effect of Thioperamide on Changes in CASNA and CBF after ID Administration of *C. pyrenoidosa*.

Male wistar rats (CASNA: saline group, *n*=5; thioperamide group, *n*=5; CBF: saline group, *n*=5; thioperamide group, *n*=5) were also used in a separate trial to determine the effect of thioperamide maleate salt (Sigma-Aldrich Corp., St Louis, MO, USA), a histamine H3 antagonist, on changes in CASNA and CBF associated with *C. pyrenoidosa* suspension. The animals were anesthetized with urethane (1 g/kg, IP), a polyethylene catheter was inserted into the right jugular vein, and 0.1ml of thioperamide (0.2mg/0.1ml in saline) was then injected intravenously (IV) as described in our previous studies (Nagai et al., 2003) with physiological saline used as the control. Thirty min after thioperamide or physiological saline injection, the *C. pyrenoidosa* was administered intraduodenally. CASNA and CBF were measured as described above.

Statistical Analysis

CASNA and CBF were measured at 5-min intervals over the entire 60-min test period and examined by digital signal processing analyses. All data are expressed as mean ± SEM. The Mann-Whitney U-Test was used to detect statistically significant differences between the values at 0 min (baseline) and at 5 min before ID administration in the *C. pyrenoidosa* and control groups for each parameter. Data are expressed as percentage of the baseline values for CASNA neural discharge and CBF because of the inter-individual variability in the pre-injection state.

TEWL values were calculated as the percentage change from Day 0. The Mann-Whitney U-test was used to determine the statistical significance of the difference between the baseline (Day 0) values of the *C. pyrenoidosa* and control groups.

Analysis of variance (ANOVA) with repeated measures was applied to evaluate group differences in CASNA, CBF and TEWL.

RESULT

Effect of *C. pyrenoidosa* on CASNA in Urethane-anesthetized Rats

In our preliminary experiment, it was observed that ID administration of 1 ml of *C. pyrenoidosa* (30 mg/250 ml) clearly depressed CASNA, but that of 1ml of either *C. pyrenoidosa* suspension (3 mg/250 ml) or *C. pyrenoidosa* (300 mg/250 ml) did not in urethane-anesthetized rats. Therefore, the effect of ID administration of 1ml *C. pyrenoidosa* suspension (30 mg/250 ml) on CASNA was examined in urethane-anesthetized rats. Figure 1 shows a representative recording image (Fig. 1A) and a graph (Fig. 1B) of CASNA after administration of *C. pyrenoidosa* and water. ID administration of *C. pyrenoidosa* gradually and markedly reduced CASNA while administration of water did not affect CASNA so much until 30 min after the administration but then elevated slightly. Baseline (0 min)

![Figure 1](image-url)
Effects of *C. pyrenoidosa* on skin health

As shown in Fig. 3, the *ad-libitum* oral intake of water did not affect TEWL values. Comparing to TEWL values after the water intake, TEWL gradually decreased after the start of *ad-libitum* oral intake of *C. pyrenoidosa*. Baseline (Day 0) values of TEWL before the start of the intake were 10.8 ± 0.4 g/m²/h (water) and 10.8 ± 0.6 g/m²/h (chlorella) (N.S.).

**Effects of *C. pyrenoidosa* on CBF**

The effect of the administration of *C. pyrenoidosa* or water on the CBF (ml/min/100 g of tissue) was examined. Figure 2 shows changes in the CBF at the proximal end of the dorsal tail skin after administration of *C. pyrenoidosa* or water, which are expressed as mean ± SEM of percentage of 0 min values. The CBF after water administration gradually lowered. In contrast, CBF after administration of *C. pyrenoidosa* slightly lowered once and then mildly elevated. Baseline (0 min) absolute value of water group was 4.19 ± 0.7 ml/min/100 g of tissue and that of chlorella group was 3.67 ± 1.2 ml/min/100 g of tissue (N.S.).

**FIGURE 2. Effects of intraduodenal injection of *Chlorella pyrenoidosa* on the cutaneous blood flow.** The time course changes in blood flow after intraduodenal injection of water or *Chlorella pyrenoidosa* are expressed as mean ± SEM of the percentages of the values at 0 min. The data (mean ± S.E.M) are expressed as the percentage of change in the values from the baseline value (n = 5). *The significant differences (P < 0.05) between the values recorded from 5–60 min after intraduodenal administrations of *Chlorella pyrenoidosa* and water were analyzed by analysis of variance with repeated measures.

Effects of *C. pyrenoidosa* on TEWL

Figure 3 shows the changes in TEWL measured in the dorsal region of the hairless rats from Day 0 prior to *ad-libitum* oral intake and from Day 1 to Day 3 after the start of the *ad-libitum* oral intake of *C. pyrenoidosa* or water given as a sole drinking water. The TEWL values were expressed as mean ± SEM of percentage of the baseline values obtained at Day 0.
Effects of *C. pyrenoidosa* on skin health

FIGURE 4. Effect of intravenous (IV) injection of thioperamide on changes in CASNA and cutaneous blood flow. (A) Changes in CASNA after to intraduodenal injection of *Chlorella pyrenoidosa* in urethane-anesthetized rats given the previous IV injection of saline or thioperamide 30 min prior to the intraduodenal injection are shown. Data (mean ± S.E.M) are expressed as the percentage of change in the values from the baseline value (n = 3). *The significant differences (P < 0.05) between the values recorded from 5–60 min after intraduodenal injection of *Chlorella pyrenoidosa* after the previous IV injection of saline or thioperamide 30 min prior to the intraduodenal injection were analyzed by analysis of variance with repeated measures. (B) Changes in blood flow after to intraduodenal injection of *Chlorella pyrenoidosa* in urethane-anesthetized rats given the previous IV injection of saline or thioperamide 30 min prior to the intraduodenal injection were analyzed by analysis of variance with repeated measures. (A) shows the effect of IV injection of thioperamide on the increase of CBF due to ID administration of 1 ml of *C. pyrenoidosa*. IV injection of 0.1 ml of saline given 30 min prior to the *C. pyrenoidosa* administration did not affect the elevation in CBF after *C. pyrenoidosa* administration. However, IV injection of 0.1ml of thioperamide given 30 min prior to the *C. pyrenoidosa* administration eliminated the elevation of CBF caused by *C. pyrenoidosa* administration and CBF gradually decreased. Baseline (0 min) absolute value of saline-group was 8.8 ± 3.8 ml/min/100 g of tissue and that of thioperamide group was 3.1 ± 0.1 ml/min/100 g of tissue (N.S.).

DISCUSSION

Our research was designed to confirm that *C. pyrenoidosa* has a positive effect on good skin condition through measurement of autonomic nerve activity and physiological response. It was demonstrated that administration of *C. pyrenoidosa* affected CASNA, CBF, and TEWL.

Our most important finding was that *C. pyrenoidosa* caused a decrease in CASNA (Fig. 1B). This decrease in CASNA is the first step in a chain that leads to increased CBF (Fig. 2). This increased CBF then leads to a subsequent decrease in TEWL (Fig. 3). The end result is improvement in all three skin health markers through an enhanced supply of oxygen and nutrients to the skin and an increase in its water-retaining ability.

We then considered what are the physiological mechanism(s) involved in control of the changes in CASNA as well as CBF and TEWL. Since the suppression of CASNA due to urea was eliminated by thioperamide (Horii et al., 2011), it is likely that histamine H3 receptor is involved in the regulation of CASNA. Thus, we examined the effect of thioperamide on the reduction in CASNA due to *C. pyrenoidosa*. When *C. pyrenoidosa* and thioperamide were administered together, CASNA no longer exhibited a decrease (Fig. 4A). This result strongly suggests that *C. pyrenoidosa* suppresses CASNA via the histamine H3 receptors.

Once we determined that the histamine H3 receptor may regulate CASNA, we considered what physiological mechanism linked CASNA to changes in CBF. An examination of the literature revealed that increases in CASNA have been shown to cause constriction of the cutaneous arterioles via the α1-adrenergic receptor (Ganong, 2003). Therefore, we propose that a decrease in CASNA caused by administration of *C. pyrenoidosa* induces vasodilatation of these cutaneous arterioles. This vasodilatation of the cutaneous arterioles is probably the key factor causing the increase in CBF observed in this study (Fig. 2). Supporting of physiological mechanism linked CASNA to CBF; when *C. pyrenoidosa* and thioperamide were administered together, CBF no longer exhibited a increase (Fig. 4B). This result suggests that *C. pyrenoidosa* administration elevated CBF via a histaminergic H3 receptor.

Histaminergic neurons are located in the tuberomammillary nucleus of the hypothalamus and project on central nervous system (Krout et al., 2002; Pillot et al., 2002). H3 receptors
which localized in the presynaptic cleft cause the inhibition of histamine release from the presynaptic histamine neuron to synaptic clefts. Therefore, a small amount of histamine in the synaptic cleft is thought to suppress histamine release via the H3 receptor (Arrang et al., 1983). The effect of \textit{C. pyrenoidosa} via H3 receptors may therefore trigger a decrease in histamine release. However we could not determine the validity of this hypothesis within this study, therefore further investigation regarding this possibility is required.

ID administration of \textit{C. pyrenoidosa} may increase the supply of oxygen and nutrients to the skin due to an increase in CBF. Previously, we observed that the administration of \textit{Lactobacillus brevis} SBC8803 suppressed CASNA, increased CBF and reduced TEWL (Horii et al., 2014). Our current study also confirmed that the oral ad-libitum administration of \textit{C. pyrenoidosa} reduced TEWL, an indicator of water-retaining ability, in the dorsal skin of hairless rats (Fig. 3). We speculate that increases in the supply of oxygen and nutrients to the skin induced by an elevation in cutaneous blood flow are important for maintaining of the stratum corneum. The end result is enhancement of the epidermal diffusion barrier induced by a reduction in TEWL. TEWL is considered to reflect the functional state of the epidermal diffusion barrier, so a reduction in TEWL indicates an improved barrier function. As a whole, our findings are consistent with the possibility that \textit{C. pyrenoidosa} given \textit{per os} increases water-retaining ability of the skin, which may be accomplished through decreased CASNA, increased CBF, and elevated supply of oxygen and nutrient to the skin.

Two limitations should be kept in mind when interpreting our results: first, the animals in the study groups were healthy, and second, all animals were young. We used healthy animals with a normal circadian rhythm because of the close relationship between circadian rhythm and the autonomic nervous system. We previously showed that the hypothalamic suprachiasmatic nucleus (SCN), the master circadian clock, is involved in the regulation of autonomic nerves (Nagai et al. 1996; Buijis et al. 2001). We furthermore previously confirmed that bilateral lesions of the SCN eliminated autonomic nerve activity and cardiovascular response to \textit{Lactobacillus johnsonii} L41 (Tanida et al. 2005b). Since the autonomic nerve activity and thermal response to odor exposure are preserved in a light period, but not in a dark period (Tanida et al. 2008), we took particular note of when experiments were conducted.

Second, the age of our sample limits the generalizability of this study. We measured CASNA, CBF and TEWL in a sample group of young, 7-week-old animals. An older sample group was not used primarily because of scheduling considerations. Accordingly, further trials should be performed on older animals to confirm our findings.

In conclusion, administration of \textit{C. pyrenoidosa} increased CASNA, increased CBF, and decreased TEWL. These findings suggest that \textit{C. pyrenoidosa} administration may increase water-retaining ability of the skin and improve wide range of skin problems through the suppression of CASNA and the elevation of CBF most likely through the involvement of histamine neurons.

**CONFLICT OF INTEREST DISCLOSURE**

The authors declare that they have no conflict of interest.

**REFERENCES**


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