The Immunological Effects of Electrolyzed Reduced Water on the *Echinostoma hortense* Infection in C57BL/6 Mice

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Received July 9, 2008; accepted December 24, 2008; published online December 26, 2008

Electrolyzed reduced water (ERW) is widely used for drinking by people in Asia. The purpose of this study was to examine the immunological effect of ERW on the immunity of animals by supplying ERW to C57BL/6 mice infected with *Echinostoma hortense* metacercariae. In the non-infected groups, interleukin (IL)-4 (p<0.001), IL-5, IL-10, IL-1β, tumor necrosis factor (TNF)-α and immunoglobulin (Ig) A expression of the group fed ERW (ERW group) increased in small intestine compared with the normal control group. In the case of infected groups, the group fed ERW (ERW+*E. hortense* group) showed the result that IL-4, IL-5, IL-10 and Ig A expression increased, but IL-1β and TNF-α (p<0.001) decreased, and the number of goblet cells (p<0.001) and helix pomatia agglutinin (HPA) positive cells increased compared with the group without feeding ERW. However, adult worm recovery rate was markedly increased (p<0.05). On the other hand, the expression of all the cytokines except IL-10 in spleen was mildly increased but not significant statistically, and there was no significant difference in the numerical changes of white blood cell (WBC). These results indicate that feeding ERW may have influence on the local immune response (Th-1 type cytokines such as IL-1β, TNF-α) in the small intestine but not on the systemic immune response.

Key words electrolyzed reduced water; *Echinostoma hortense*; local immune response; worm expulsion

Recently, as the consumption of electrolyzed reduced water (ERW) is increasing socially, the necessity of studies addressing the mechanism of various ERW effects in vivo has been suggested. It has been reported that ERW mediates reactions similar to superoxide dismutase (SOD) and thus it could remove reactive oxygen species (ROS). ERW has been proven experimentally to suppress DNA damage.1) The ROS scavenging effect of ERW has been known to be a major action mechanism in vivo and in vitro. ROS have been reported to be a major cause of cancer, mutation, etc. by oxidative stress in vivo.2,3) Besides the removal of ROS, ERW is thought to be related with immune response, although studies on this association are extremely rare.

Generally, when infected with an intestinal helminth, the host activates killing mechanisms such as releasing proteases or anti-oxidant enzymes.4) In addition, the reaction of adipose cells, goblet cells, eosinophils, and neutrophils could also be considered a counteraction, parasites protect themselves against oxidant damage through anti-inflammatory mechanisms such as releasing proteases or anti-oxidant enzymes.5) In addition, the reaction of adipose cells, goblet cells, eosinophils, and neutrophils could also be considered a major mechanism in parasite expulsion, and overall, this response is associated with cytokines.5,6) It has been reported that progression of infectious disease, including parasitic disease, often depends on the dominance of Th1 or Th2 type cytokines,7) the Th1 response is dominant in intracellular parasite infection,8) and Th2 type is dominant in extracellular parasite infection.9) *Echinostoma hortense* is an extracellular parasite that lives in the small intestine, and belongs to the family Echinostomatidae. *E. hortense* was reported by Asada for the first time in 1926. Infections of *E. hortense* have been reported continuously in Korea as well as other countries.

*E. hortense* develops from an egg to a miracidium, lives as a cercaria in the first intermediate host, and then develops into a metacercaria upon invading the second intermediate host. The metacercaria grow into mature adult worms in a definitive host such as rats or dogs after approximately 10 d.10) The growth of *E. hortense* is interrupted and destroyed by the protective response of the host, and thus, is expelled from the host after a certain infection period.11) In this process, worm expulsion by the host is closely associated with the enhancement of a Th2-mediated response, hyperproliferation of goblet cells in the small intestinal mucosa, and the increased secretion of mucin.12,13)

Until recently, studies on ERW were mainly in association with the ROS scavenging effect of ERW. However the effect of ERW may have additional mechanisms of disease improvement, which must be elucidated by animal or clinical studies. In association with the in vivo effect of ERW, we examined the effect of ERW on the activation of Th1 and Th2 cytokines, the expression of goblet cells, and worm expulsion from the aspect of immunology after infecting C57BL/6 mice with *E. hortense*.

MATERIALS AND METHODS

Materials Both the filtered water (FW) and ERW were produced by the continuously electrolyzing apparatus (BTM-700, Biontech, Korea) under different conditions. Main source of water was a tap water. FW was produced only by physical filtering using a sedimentation filter and an activated carbon filter. ERW was prepared by physical filtering followed by electrolysis and collected in a cell equipped with a cathode platinum-coated titanium electrode (0.9±0.1 A, 1—21/min at 2 kgf/min). ERW was adjusted to pH 10.05±0.05

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and oxidative-reduction potential of $-395.0 \pm 16.5$ mV. The electrical conductivity (EC) of ERW was $223 \pm 5.5$ $\mu$S, and dissolved oxygen (DO) was $4.82 \pm 0.11$ mg/L. Glass bottles were used for water feeding. ERW was changed three times a day.

**Animals**  
Animal experiments were approved by and conducted under the guidelines of the Animal Care and Use established by The Ethics Review Committee of Yonsei Wonju College of Medicine, Wonju, Gangwon, Korea. The experimental animal was a 4 weeks old female C57BL/6 mice (Orientbio Inc., Seongnam, Korea). They were acclimated for one week and reared under the condition of the mice (Orientbio Inc., Seongnam, Korea). The mental animals were divided into 4 groups 10 in each; normal control group (provided filtered water without infection), the infected control group (provided filtered water with *E. hortense* infection), the ERW group (provided ERW, without infection), and ERW + *E. hortense* group (provided ERW with *E. hortense* infection). The two water types were provided to each group from five weeks prior to infection, to two weeks after infection.

**Infection of *E. hortense* Metacercaria**  
Fifteen metacercaria per mouse were used to orally infect the confirmed control and ERW + *E. hortense* groups. For infection of *E. hortense* metacercaria, the anal area of loaches infected with *E. hortense* was dissected, pressed by two slides, and then a tissue containing 15 metacercaria was verified by light microscope was administered orally to each mouse.

**Immunohistochemical Staining of IL-4, IL-10, and TNF-α in Small Intestine Tissues**  
Murine small intestine samples sectioned 4 $\mu$m in thickness were treated with 5% rabbit normal serum for 20 min. After washing with 0.01 M phosphate buffer solution (PBS, pH 7.4), monoclonal rat anti-mouse IL-10 antibody diluted to 1:30 (PharMingen, San Diego, CA, U.S.A.) or biotinylated goat anti-rabbit IgG (IgG) diluted to 1:200 (Vector Laboratories, Burlingame, CA, U.S.A.) was added and reacted at 37 °C for 30 min. After washing with PBS, streptavidin-conjugated peroxidase (Dako, Carpinteria, CA, U.S.A.) was added and reacted at 37 °C for 20 min. After washing with PBS, the samples were stained with 3, 3'-diaminobenzidine (DAB), and counter stained with Mayer's hematoxylin.

**Immunohistochemical Staining of IgA within Small Intestine Tissues**  
To murine small intestine sample section 4 $\mu$m in thickness, FITC-conjugated rat anti-mouse IgA (PharMingen, San Diego, CA, U.S.A.) was added and reacted at room temperature for 30 min. After washing with PBS, the sections were sealed with glycerin jelly, and examined under a light microscope.

**The Assessment of Cytokine Expression in the Spleen by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**  
The changes in cytokine mRNA expression on the spleen were determined by RT-PCR. Total RNA was extracted from spleens of mice using Trizol reagent (Invitrogen, CA, U.S.A., Cat. No. 15596-018) according to manufacturer’s instruction. Four micrograms total RNA extracted from the spleen was mixed with 1 $\mu$l 200 pmol of oligo dT$_{15}$ Primer (Promega, Madison, WI, U.S.A.), incubated at 70 °C for 5 min, and mixed with AccuPower® RT-PreMix (Bioneer, Daejeon, Korea). The total volume was adjusted to 50 $\mu$l with sterile 0.01% DEPC distilled water, and cDNA was synthesized by incubation at 42 °C for 60 min. And subsequently at 94 °C for 5 min. For PCR reaction, 4 $\mu$l cDNA, 5 $\mu$l dNTP (iNtRON Biotechnology, Sungnam, Korea), 1 $\mu$l of 100 pm/μl primer set (Table 1), 0.25 U Taq polymerase (iNtRON Biotechnology, Sungnam, Korea), and 5 $\mu$l 10×PCR buffer (iNtRON Biotechnology, Sungnam, Korea) were added, and the volume was adjusted to 50 $\mu$l by adding distilled water. Regarding PCR conditions, denaturation was performed at 94 °C for 30 s, annealing was performed depending on primers at 60—64 °C for 30 s, and extension was performed at 74 °C for 1 min. The PCR product length after amplification of the sense and antisense primer sequences is shown in Table 1. The PCR product was assessed by 2% agarose gel

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**Table 1. Primer Sequences and Amplified Fragment Sizes of the Cytokines Used in This Study**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplified fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>Sense TCT CTA GAT CAT GGG CAT TTT GAA CGA GGTCC</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Antisense TGC ATG ATG CTC TTT AGG CTG TCC</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>Sense ATG ACT GTG CCT CTG TCG GTG CAG C</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>Antisense CTG TTT TTT CTG GAG TAA ACT GGG G</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Sense GCT ACC TGT GTT TTT CCC GTG G</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>Antisense TGG TCG TGG CCT GGT TCT CCT TG</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense GGC AGG TCT ACT TTA GAG TCA TTG C</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Antisense ACA TGC GAG CCT CCA GTG AAT TCG G</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Sense CAC TGC TAT GCT GCC TGC TCT T</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td>Antisense TCT TCA CCT GCT CCA CTG CCT T</td>
<td></td>
</tr>
</tbody>
</table>
The tissue of the upper small intestine (1.5 cm in length, fixed with Carnoy’s solution, and sectioned 4 μm in thickness. The sections were stained with periodic acid Schif (PAS) and counterstained with hematoxylin. The number of goblet cells in ten villus-crypt units (VCU) was counted under a light microscope.

Examination of Goblet Cells by Lectin Histochemical Staining To examine the functional status of activated goblet cells in the small intestine of mice, the method described by Ishikawa, lectin histochemical staining, was performed.14,15) The tissue of the upper small intestine (1.5 cm in length from 1 cm under the stomach) was fixed with Carnoy’s solution, embedded in paraffin, and sectioned 4 μm in thickness. The paraffin sections were deparaffinized with xylene and hydrated with graded alcohol. To inactivate endogenous peroxidase activity, the samples were treated with methanol containing 0.3% H2O2 for 20 min, washed 3× with 0.01 M PBS (pH 7.4) for 2 min, blocked with PBS containing 1% bovine serum albumin (BSA), and washed with PBS. In a moist chamber, 25 μg/ml biotinylated helix pomatia agglutinin (HPA, Sigma L-6512, Sigma, St. Louis, MO, U.S.A., 1 mg) was added to the sample, incubated for 2 h, washed with PBS, treated with streptavidin-horseradish peroxidase (HRP) conjugate (Zymed Laboratories, San Francisco, CA, U.S.A., 2.5 mg/2 ml), and incubated in PBS (1 : 300) for 2 h. The samples were stained for 10 min using a liquid DAB-plus substrate kit (Zymed Laboratories, San Francisco, CA, U.S.A., 100 μl), and counterstained with Mayer’s hematoxylin for 10 s. After washing with tap water, the samples were treated with ethanol for dehydration, with xylene for clearing, and mounted with Canada balsam.

The Expression of Cytokine mRNA and IgA in the Small Intestine Mucosa. Th2 Type Cytokines (IL-4, IL-5, and IL-10) In the case of IL-4 expression, 73.3±11.7 cells/10 VCU (villus-crypt unit) was observed for the normal control group, while 146.5±7.6 cells/10 VCU, 116.3±3.2 cells/10 VCU, and 128.5±3.5 cells/10 VCU were observed for the ERW, infected control, and ERW + E. hortense groups, respectively. The two groups treated with ERW showed a tendency to have greater expression levels than the control groups. Among the groups, the ERW group exhibited the highest value and showed a statistically significant difference compared to the normal control group (p < 0.01). In the case of IL-5 expression, 53.3±14.5 cells/10 VCU, 212.3±32.6 cells/10 VCU, 61.7±19.2 cells/10 VCU, and 247.8±91.5 cells/10 VCU were observed for the normal control, infected control, ERW, and ERW + E. hortense groups, respectively. In the case of IL-10 expression, the normal control group was shown to be 68.8±8.8 cells/10 VCU, followed by 161.3±48.8 cells/10 VCU for the ERW group, 92.9±2.9 cells/10 VCU for the infected control group, and 134.5±20 cells/10 VCU for the ERW + E. hortense group. All of the cytokines examined showed increased expressions in the groups fed ERW compared with control groups irrespective of infection. However, there was no significant difference, except for IL-4 expression, between the non-infection groups (Fig. 1).

Pro-inflammatory Cytokines IL-1β, TNF-α, and IgA Concerning IL-1β expression, the normal control group was 47.5±7.2 cells/10 VCU, the ERW group was 70.0±0.0 cells/10 VCU, the infected control group was 193.5±40.2 cells/10 VCU, and the ERW + E. hortense was 146.7±42.3 cells/10 VCU. Concerning TNF-α expression, the normal control group was 26.3±26.3 cells/10 VCU, while the ERW group was 112.5±65.5 cells/10 VCU, followed by 177.9±26.3 cells/10 VCU for the infected control group, and 17.3±17.3 cells/10 VCU for the ERW + E. hortense group. IL-1β and TNF-α in the two groups supplied ERW showed lower expression in comparison with their control groups. In particular, TNF-α expression of the ERW + E. hortense group was significantly decreased compared to the infected control group (p < 0.01). Concerning IgA expression, the normal control group was 99.3±21.2 cells/10 VCU, the ERW group was 166.4±39.5 cells/10 VCU, the infected control

RESULTS

The Number of Leukocytes In all groups, blood was collected using a heparinized capillary tube from the orbital vein of the mice, smeared on the slide to form a blood film, and stained with Wright solution (Muto Pure Chemicals, Tokyo, Japan). Using a light microscope, the basophils, neutrophils, lymphocytes, and monocytes present per 100 leukocytes were counted separately.

The Worm Recovery Rate After two weeks of infection, the mice of the infected control group and the ERW + E. hortense group were sacrificed, and adult worms were collected from the upper, middle, and lower area of the small intestine. The worm recovery rate was calculated by the percentage (%) of recovered adult worms relative to the number of metacercaria infected mice.

Statistical Analysis The statistical significance among the groups was analyzed by Student t-test. Concerning tissue sample cases, three tissue samples per group were prepared. Significance was based on a p < 0.05 level.

Data are expressed as means ± S.D. ** p < 0.01.

Fig. 1. The Number of IL-4, IL-5, IL-10, IL-1β, TNF-α and IgA Expressing Cells per 10 Villus-Crypt Units (VCU) in the Small Intestine Villi of C57BL/6 Mice by DBA (IL-5 and IL-1β) and Immunofluorescent Staining (IL-4, IL-5, and TNF-α)
group was $176.9 \pm 34.7$ cells/10 VCU, and the ERW + *E. hortense* group was $278.3 \pm 41.8$ cells/10 VCU. Although it was not significant, the two ERW groups were shown to have higher IgA levels than their control groups (Fig. 1).

The Expression of Cytokine mRNA in the Spleen. Th2 Type Cytokines (IL-4, IL-5, and IL-10) In the case of IL-4, expression in the normal control group was $877.7 \pm 192.7$, $912.3 \pm 29.3$ in the infected control group, $986.7 \pm 44.3$ in the ERW group, and $938.8 \pm 97.7$ in the ERW + *E. hortense* group. In the case of IL-5, the normal control group expression was $1331.4 \pm 176.5$, $1706.3 \pm 66.1$ for the infected control group, $1647.1 \pm 162.5$ for the ERW group, and $1872.7 \pm 3.2$ for the ERW + *E. hortense* group. IL-10 expression in the normal control group was $1293.6 \pm 776.5$ in the ERW group, and $1567.1 \pm 68.3$ in the ERW + *E. hortense* group. According to these results, IL-4 and IL-5 were expressed slightly higher in the two ERW groups than in the control groups. However, expression of IL-10 mRNA was highest in the normal control group, while the two ERW groups showed lower expression than the two control groups. Nevertheless, the differences between IL-4, IL-5 and IL-10 were not statistically significant (Fig. 2).

Pro-inflammatory Cytokines (IL-1β and TNF-α) IL-1β expression was $776.5 \pm 118.4$ in the normal control group, $773.5 \pm 94.2$ in the infected control group, $881.4 \pm 90.7$ in the ERW group, and $1116.5 \pm 64.2$ in the ERW + *E. hortense* group. TNF-α levels were $490.3 \pm 84.5$ in the normal control group, $543.4 \pm 56.2$ in the infected control group, $501.5 \pm 58.0$ in the ERW group, and $614.8 \pm 28.7$ in the ERW + *E. hortense* group. The expression of IL-1β and TNF-α mRNA in the spleen of the ERW groups was shown to be slightly higher than their control groups, but not at a significant level (Fig. 2).

Goblet Cell Expression in the Small Intestine Mucosa Concerning the number of goblet cells expressed as determined by PAS staining, the normal control group had $47.5 \pm 2.7$, the ERW group had $60.6 \pm 4.9$, the infected control group had $90.9 \pm 14.0$, and the ERW + *E. hortense* group had $194.1 \pm 11.4$. According to this result, the number of goblet cells increased after *E. hortense* infection, while the ERW (p < 0.001) and ERW + *E. hortense* groups (p < 0.001), had significantly increased goblet cell numbers in comparison with their control groups (Fig. 3).

Goblet Cell Expression Assessed by Lectin Immunohistochemical Staining The number of HPA positive goblet cells by lectin staining was $82.4 \pm 7.3$ for the normal control group, $98.5 \pm 15.0$ for the ERW group, $147.0 \pm 4.4$ for the infected control group, and was $156.8 \pm 9.2$ for the ERW + *E. hortense* group. Similar to the result of PAS staining, the expression of HPA positive cells was increased more after infection although the increase was not significant (Fig. 3).

The Change of the Number of Leukocytes The relative numbers of cells in the normal control group were as follows: eosinophil $1.5 \pm 1.0$, neutrophil $14.5 \pm 4.8$, lymphocyte $83.8 \pm 4.6$, and monocyte $0.1 \pm 0.3$. The relative cell numbers in the ERW group were as follows: eosinophil $0.5 \pm 0.8$, neutrophil $9.4 \pm 3.3$, lymphocyte $89.9 \pm 3.7$, and monocyte $0.2 \pm 0.4$. In the case of the non-infection groups, the number of eosinophils (p < 0.05) and neutrophils (p < 0.01) in the ERW group decreased, while lymphocytes (p < 0.01) increased significantly in comparison with the normal control group. On the other hand, the infected control group showed the following numbers: eosinophil $1.5 \pm 1.6$, neutrophil $8.4 \pm 3.2$, lymphocyte $90.0 \pm 3.2$, and monocyte $0.2 \pm 0.0$. In the ERW + *E. hortense* group, cells counts were as follows: eosinophil $1.3 \pm 1.9$, neutrophil $8.5 \pm 3.8$, lymphocyte $90.0 \pm 4.8$, and monocyte $0.2 \pm 0.4$. Differences between the uninfected and infected groups were not statistically significant (Fig. 4).

The Worm Recovery Rate To examine the worm recovery rate, the mice of the infection groups were sacrificed two weeks after infection, and adult worms were collected from the small intestine. As a result, the worm recovery rate was $18.3 \pm 5.1\%$ in the infected control group and $36.6 \pm 4.8\%$ in the ERW + *E. hortense* group. The ERW + *E. hortense* group showed a significantly higher worm recovery rate than the infected control group (p < 0.05) (Fig. 5).
hosts.16—18) For example, in the case of nematodes, immune responses are different depending on the parasites and asite loss directly or indirectly is very complex, and the im-
mune system and seems to be important in the expulsion of adult worm development.19) Our study of Th2 cytokine (IL-4, IL-5 and IL-10) expression after E. hortense infection showed similar results to previous cases of gastrointestinal parasite infection. Although it was not statistically signif-
ificant, Th2 cytokines were expressed at higher levels in the in-
grouped than the normal control group. Moreover, Th2 cytokines of the ERW+E. hortense group were increased more than in the infected control group (Fig. 1). However, despite the elevation of Th2 type cytokine expression, worm expulsion was delayed, which may indicate that the Th2 cy-
tokine expression was not high enough to have a protective effect. Furthermore, the reduction of Th1 cytokines (TNF-α and IL-1β) and the immunological effect of ERW may have had an effect on the Th2-mediated immune response in the small intestine. Concerning the studies explaining the relationship of Th2 cytokines and worm expulsion, Scales et al. reported that even if TNF-α, IL-4, and IL-5 were reduced by blocking of an inducible costimulator (ICOS), it did not ef-
fect the expulsion of Trichinella spiralis adult worms.20) Artis et al.21) have also reported that in vivo blockade of TNF-α in normally resistant mice, while not altering IL-4, IL-5 or IL-
13 production in the draining lymph node, significantly de-
layed worm expulsion for the duration of treatment. These reports suggest that Th2 cytokines are not essential for the worm expulsion.

Parasite infection increases the number of goblet cells and induces a qualitative change of mucin secretion in the small intestine. Such changes mediated by the CD4+ Th2 immune response generally accelerate the expulsion of worms.14,22) However, Chai et al.23) have reported that mastocytosis and goblet cell hyperplasia are local immune responses against N. seoulensis, which play a major role in the host defense and worm expulsion. In addition, Ishikawa14) has reported an al-
teration of sugar residues present on goblet cell mucins. In particular, the strong expression of terminal N-acetyl-D-galactosamine in the small intestine is regulated by the host’s immune system and seems to be important in the expulsion of N. brasiiliensis.19) In our study, expression of HPA positive goblet cells was higher in the infected control group than in the normal control group and was higher in the ERW+E. hortense group than in the infected control group although not at significant levels (Fig. 3). The number of goblet cells expressed after infection with E. hortense increased in the ERW+E. hortense group compared with the infected control group (p<0.001) (Fig. 3). However, the worm recovery rate of the ERW+E. hortense group was higher than the infected control group (p<0.05), which was due to the delay of worm expulsion (Fig. 5). Such contradictory results of goblet cell hyperplasia and the expulsion delay is supported by the re-
port of Garside et al. that goblet cell hyperplasia and the in-
crease of mucin secretion are not essential factors for the protective immune response in all gastrointestinal helminth infections.24)

The expression of IgA in the small intestine mucosa as measured by immunofluorescent staining increased in the in-
fected as well as the non-infected groups. In particular, the ERW+E. hortense group showed a markedly increased IgA expression in comparison with the infected control group (Fig. 1). According to the other researchers, IgA is secreted in large quantities in response to parasite infection to protect
the cell membrane of the mucosal epithelium, recognize and interact with antigens produced by parasites, help macrophage phagocytosis, and accelerate worm expulsion.\textsuperscript{25,26} Such mechanisms explain the elevation of IgA expression in C57BL/6 mice infected with \textit{E. hortense}, and it is thought that ERW stimulates further IgA reactions.

Intestinal pathology after gastrointestinal parasite infection is usually mediated by Th1 responses, while infection with gastrointestinal parasites elicits potent Th2 responses, which ultimately result in parasite expulsion. In addition, immune expulsion of nematodes is occasionally accompanied by inflammatory reaction in the small intestine.\textsuperscript{16} In our experiments, the expression of IL-1\(\beta\) and TNF-\(\alpha\) involved with Th1 cytokines was increased in comparison with the normal control group after \textit{E. hortense} infection. Nevertheless, that of the ERW+E. \textit{hortense} group was lower than that of the infected control group, particularly TNF-\(\alpha\) expression (\(p<0.001\)) (Fig. 1). This result suggests the possibility that ERW may mediate effects not only on small intestine physiology, but also local immunity. In addition, TNF-\(\alpha\) stimulates the production of inducible NO synthase (iNOS) consequently elevating NO levels in the small intestine, which induces Th2 reaction. Therefore, TNF-\(\alpha\) plays a role of accelerating worm expulsion. iNOS produced in the small intestine plays an important role in the maintenance of epithelial layer homeostasis in the small intestine and host protection. The overproduction of NO acts as an important pathogenesis of inflammatory conditions including tissue damage.\textsuperscript{20} Such studies show that the reduction of NO production due to the reduction of TNF-\(\alpha\) may contribute to the improvement of enteropathy. In addition, if excessive Th1 response could be suppressed without losing the protective functions mediated by Th2 cytokines, it may be beneficial to the host. Therefore, based on the result of our study upon ERW treatment, Th2 cytokines were elevated while IL-1\(\beta\) and TNF-\(\alpha\) were reduced. This finding suggests the possibility of ERW as a TNF-\(\alpha\) or iNOS inhibitor. Furthermore, it was determined that in the cases of parasite infection that do not induce severe pathological damage in the host, ERW may help improve the pathology in the infection area.

In association with the pathological process caused by parasite infection in the small intestine, production of reactive oxygen species to antagonize parasites may be involved as another factor that affects the intestinal environment. During the period when parasite killing by the host is occurring, activated host leukocytes produce a large quantity of reactive oxygen species (ROSs), such as superoxide radical anion and hydroxyl radicals. To counteract these ROSs, parasites produce antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, cytochrome \(c\) peroxidase, and glutathione \(S\)-transferase, thus preventing the peroxidation of their surface membrane.\textsuperscript{4,28} In our experiments, although the Th2 response was not decreased, worm expulsion was delayed. This is speculated to be due to the partial improvement of the parasite environment through the removal of reactive oxygen species by ERW. In support of this hypothesis, Vorobjeva\textsuperscript{29} has reported that electrolyzed reduced water maintains an oxidation-reduction potential (ORP) level suitable to the growth of intestinal anaerobic bacteria, and thus may mediate a positive effect on human intestinal bacteria. ERW is water that does not contain special components, but does have a characteristic high pH and ORP, and it shows a positive effect of the removal of reactive oxygen species and other diverse clinical effects in \textit{in vivo}. For example, Lee \textit{et al.} (2006) reported that ERW protects DNA, RNA, and protein from oxidative injury, while Kim M. J. and Kim H. K.\textsuperscript{10} and Kim \textit{et al.}\textsuperscript{31} reported anti-diabetic effect using animal models. In addition, Huang \textit{et al.}\textsuperscript{22} have reported that in end-stage renal disease patients, ERW reduced hemodialysis-induced oxidative stress, which suggests a potential clinical application. The mechanism of the effect of ERW shown in animal experiments has not been elucidated. But the removal of reactive oxygen species has been accepted as a major mechanism, which may also improve the intestinal environment and provide advantageous conditions against the immune evasion of parasites.

In conclusion, the immunological response of C57BL/6 mice infected with \textit{E. hortense} was similar to the immunological response induced by other gastrointestinal parasite infections. However, in the mice supplied with ERW, activation of Th1 cytokines was suppressed and worm expulsion was delayed, while activation of Th2 cytokines was maintained in the small intestine. The results indicate that ERW affects the local immune response induced by gastrointestinal parasite infection. Moreover, the marked decrease of IL-1\(\beta\) and TNF-\(\alpha\) expression by drinking ERW is expected to help prevent the enteropathy deterioration by parasite infection. However, further studies are required to define the immunological effects and mechanisms of ERW \textit{in vivo} because the protective and pathological responses may vary according to the characteristics and developing stages of the infecting parasite and host.

REFERENCES