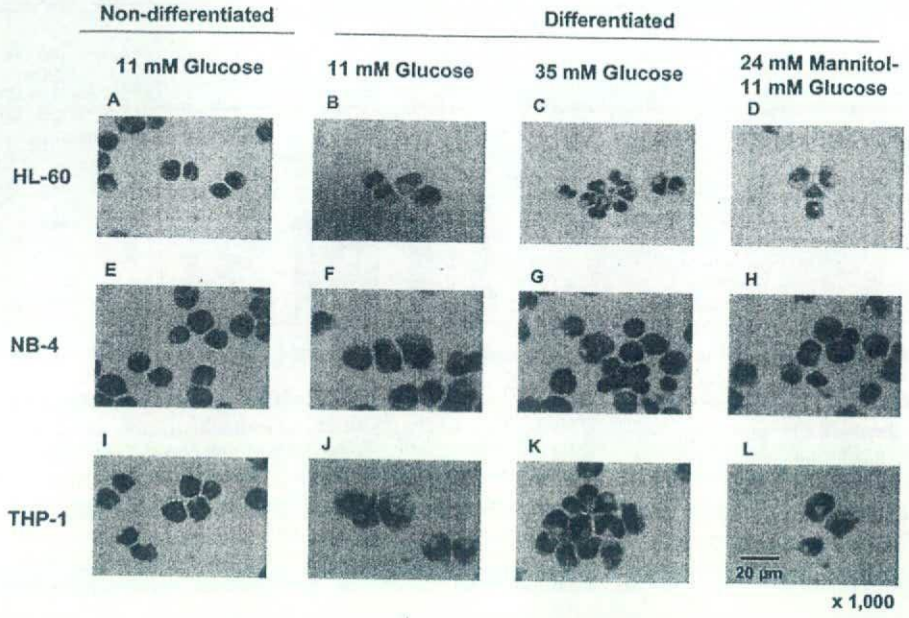

Figure 1. Morphological characteristics of non-differentiated and differentiated HL-60, NB-4, and THP-1 cells cultured with the different concentration of glucose. The cell lines were maintained in RPMI 1640 medium containing 11 mM glucose (A: HL-60, E: NB-4, I: THP-1). Differentiated cells were prepared by treatment with 1 μM ATRA for 2 days (dHL-60: B–D and dNB-4: F–H), or with 1 μM ATRA and 1 ng/mL GM-CSF for 2 days (dTHP-1: J–L). The differentiated cells were further cultured for 4 days with 11 mM glucose (B, F, and J), 35 mM glucose (C, G, and K), and 24 mM mannitol-11 mM glucose (D, H, and L). All cells used were stained with Diff-Quik.

of cells in aggregates = [aggregated cells/total cells] × 100. Each individual experiment was repeated at least three times.

2.6 NBT reducing assay

The cells ($0.4-1 \times 10^6$) were centrifuged at $700 \times g$ for 5 min, suspended with 1 mL PBS containing 2 mg/mL of NBT and 10 ng/mL of PMA, and incubated for 60 min at 37°C. The reaction was terminated by the addition of 0.4 mL of 2 N HCl and kept cooling on ice for 30 min. After centrifugation at $700 \times g$ for 5 min, the formazan deposits in the pellets were dissolved with 1 mL of DMSO, and the absorbance at 540 nm was measured. Each individual experiment was repeated at least four times. In some experiments, after NBT treatments with or without stimulation of PMA, the formazan-stained or non-stained cells were directly observed under a phase contrast microscope.

2.7 Phagocytosis of yeast by differentiated cells

Yeast particles (*Saccharomyces cerevisiae*; Sigma-Aldrich, Missouri, USA) were added to the suspension of differentiated cells in HBSS containing 5% fresh human serum and left in contact with the cells for 30 min at 37°C [27]. The final concentrations of yeast and differentiated cells were 6.25×10^7 particles/mL and 2.5×10^6 cells/mL, respectively. After centrifugation, the cell pellets were stained with Ziehl's carbolfuchsin solution (Sigma-Aldrich). The yeast particles outside the cells were stained red, and the yeast particles completely ingested by the cells were protected from taking up the stain. The cells containing unstained yeast particles were considered as cells capable of phagocytosis.

2.8 Statistical analysis

Data are presented as mean ± SD. Differences between mean values were determined by ANOVA. Differences with $p < 0.05$ were considered statistically significant.

3 Results

Three human myeloid leukemia cell lines, HL-60, NB-4, and THP-1, which were differentiated with ATRA alone or with ATRA and GM-CSF in the cell-maintaining medium containing 11 mM glucose for 2 days, showed PMN-like cell morphologies and the ratio of cytoplasm to nucleus was increased (Figs. 1A, B, E, F, I, and J). These differentiated cells, dHL-60, dNB-4, and dTHP-1, acquired NBT reducing abilities and their CD11b expressions were increased by FACS analysis (data not shown). By further treatment of the differentiated cells with 35 mM glucose or 24 mM manni-

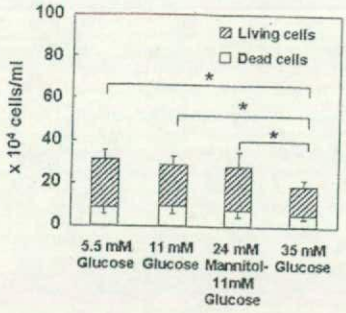

Figure 2. Effect of glucose on the cell concentration of dHL-60. After differentiation, the cells were exposed to the conditions of 5.5 mM glucose, 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The number of living and dead cells was determined by trypan blue dye exclusion under a phase contrast microscope and the sum is shown as the cell concentration (cells/mL). Data are mean ± SD from three or more independent experiments performed in quadruplicate. *, $p < 0.005$.

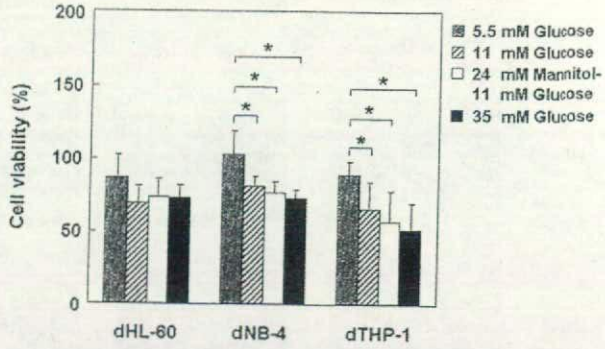

Figure 3. Effect of glucose on the cell viabilities of dHL-60, dNB-4, and dTHP-1. After differentiation, the cells were exposed to 5.5 mM glucose, 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The numbers of living and dead cells were determined by trypan blue exclusion under the phase contrast microscope. The ratio of living/dead cells was estimated as "cell viability". Data are mean ± SD from three or more independent experiments performed in quadruplicate. *, $p < 0.005$.

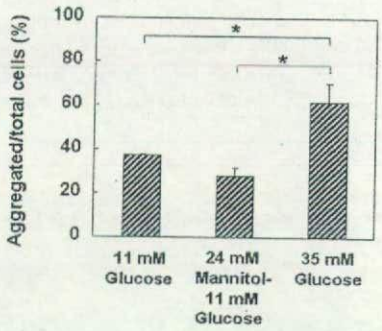

Figure 4. Effect of glucose on the cellular aggregation of dHL-60. The cells were exposed to 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The numbers of aggregated and total cells were counted under microscope and the percentage was determined. Data are mean ± SD from three to six replicate cultures. Each individual experiment was repeated a minimum of three times. *, $p < 0.05$.

tol-11 mM glucose for 4 days, there were little morphological differences between those glucose- and mannitol-treated cells (Figs. 1C, D, G, H, K, and L), and the cellular fragility under high glucose condition with 35 mM was reduced in the dHL-60 and dTHP-1 cells after PMA stimulation as described later.

In healthy human blood, the glucose concentration is in the range of 5–6 mM (*in vivo*), but the established cell lines such as HL-60, THP-1, and NB-4 were usually maintained with 11 mM glucose in RPMI 1640 medium (*in vitro*). Therefore, we first examined the effects of three different concentration of glucose, 5.5 mM (physiologic concentration), 11 mM (*in vitro* cell-maintaining concentration), and 35 mM (high concentration), on the differentiated cells. In the cell concentration of dHL-60 (Fig. 2), no significant difference was found among the differentiated cells treated with 5.5 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose during the cultures for 4 days, whereas the cell concentration in dHL-60 treated with 35 mM glucose decreased approximately up to 65% of those in cells treated with the other concentration of glucose ($p < 0.005$). No effect of dNB-4 and dTHP-1 cells by treatment with 35 mM glucose for 4 days on the cell concentration was found (data not shown). Cell viabilities (a ratio of living/dead cells) of dNB-4 and dTHP-1 cultured for 4 days with 5.5 mM glucose were significantly increased when compared with those of the other concentrations of glucose (Fig. 3, $p < 0.05$), but there was no significant difference among the viability of dHL-60 cells treated with any different concentration of glucose. These results suggest that the culture for 4 days with higher glucose concentration (up to 35 mM) did not induce mortal damages such as cytotoxicity in those cell lines.

By treatment of dHL-60 with 35 mM glucose for 4 days, the cellular aggregation was increased (Fig. 4). The mean value of aggregation under the high glucose condition with 35 mM was estimated to be 62.1 ± 8.4%, while the corresponding values under the conditions with 11 mM glucose and 24 mM mannitol-11 mM glucose were 37.3 ± 0.3% and 27.8 ± 4.0%, respectively. Statistical analysis revealed that the high glucose condition with 35 mM significantly enhanced the cellular aggregation ($p < 0.05$). Further PMA stimulation did not affect the cell aggregation of dHL-60 cells (data not shown). The dNB-4 and dTHP-1 cells showed no differences in the cell aggregation among treatments with 35 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose. However, additional PMA stimulation of dNB-4 and dTHP-1 cells treated with 35 mM glucose promoted the cellular aggregations (data not shown).

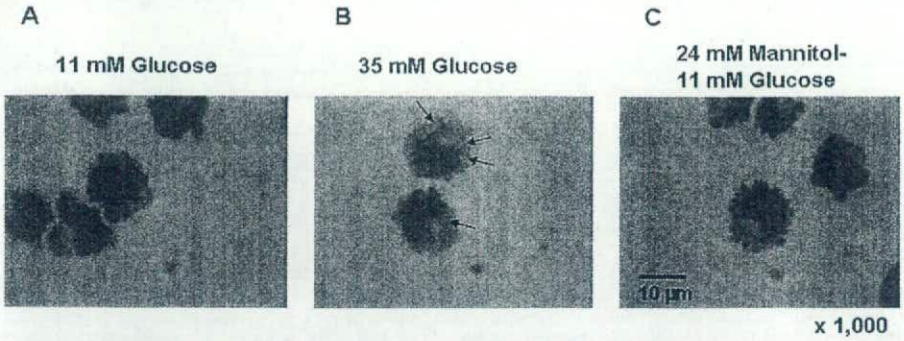

Figure 5. Effect of glucose on the intracellular vacuolation of dNB-4. The cells were exposed to 11 mM glucose (A), 35 mM glucose (B), and 24 mM mannitol-11 mM glucose (C) for 4 days. After stimulation with 10 ng/mL of PMA for 10 min, the exposed cells were stained with Diff-Quik. Arrows show cytoplasmic vacuoles.

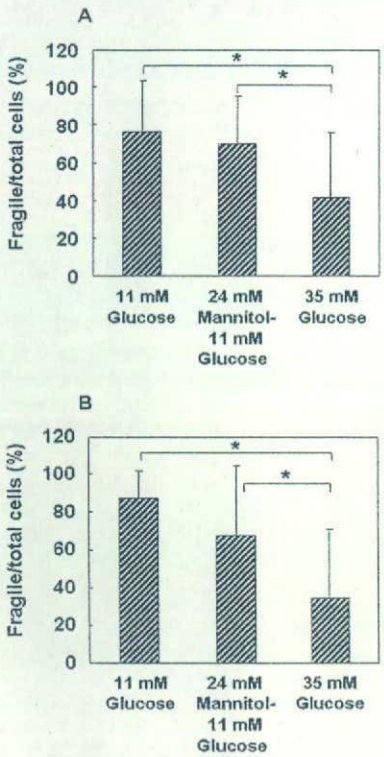

Figure 6. Effect of glucose on the cellular fragility of dHL-60 (A) and dTHP-1 (B). The cells were exposed to 11 mM glucose, 24 mM mannitol-11 mM glucose, and 35 mM glucose for 4 days. After stimulation with 10 ng/mL of PMA for 10 min, the exposed cells were stained with Diff-Quik and counted. The percentage of the cellular fragility was estimated by equation in Section 2. Data are mean ± SD from three to six replicate cultures. Each individual experiment was repeated a minimum of three times. *, $p < 0.02$.

By PMA stimulation after incubation with 35 mM glucose for 4 days (arrows in Fig. 5), the intracellular vacuolation of dNB-4 cells was induced. An equal amount of 24 mM mannitol-11 mM glucose did not enlarge vacuoles, suggesting that osmotic pressure did not affect such vacuolation. When the dNB-4 cells were not stimulated with PMA, no difference of vacuolation was observed among the cells treated with 35 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose.

Since many fragile cells of dHL-60 and dTHP-1 were observed by PMA stimulation, the effect of glucose on the cellular fragility after PMA stimulation was examined. In dHL-60 and dTHP-1 cells, the fraction of fragile cells was reduced in the cultures with 35 mM glucose compared to the cultures with 11 mM glucose and 24 mM mannitol-11 mM glucose for 4 days (Fig. 6, $p < 0.02$). In both dHL-60 and dTHP-1 cells, the fragilities due to osmotic shock were not observed as shown in treatment with 24 mM mannitol-11 mM glucose. The suppressions of cellular fragility by the cultures with 35 mM glucose were observed only when the cells were triggered with PMA.

Production of reactive-oxygen species (ROS), especially superoxide anion, was determined by NBT reducing assay in cells cultured with different concentration of glucose. In all three differentiated cells cultured with 11 mM glucose for 24 h, the superoxide production by PMA stimulation were markedly increased compared to the culture with 5.5 mM glucose (Fig. 7A, $p < 0.0002$). In dHL-60 cultured with 35 mM glucose for 24 h, the superoxide production of the cells by PMA stimulation was1.5-fold increased compared to the cultures with 11 mM as well as 5.5 mM glucose (Fig. 7B, $p < 0.0001$). However, the short-term treatment for 5 h with 35 mM glucose did not affect the superoxide generation of dHL-60 (Fig. 7C). The superoxide generation of dNB-4 and dTHP-1 cells in culture with 35 mM glucose for 24 h, 11 mM glucose, and 24 mM mannitol-11 mM glucose was significantly increased compared with 5.5 mM glucose (Fig. 7B, $p < 0.0001$). The induction of superoxide production was not observed when these differentiated cells were cultured with concentrations of glucose without the PMA stimulation (Fig. 7A, and the microscopic observation of dHL-60 cells treated with 35 mM glucose as a representative in Fig. 7D).

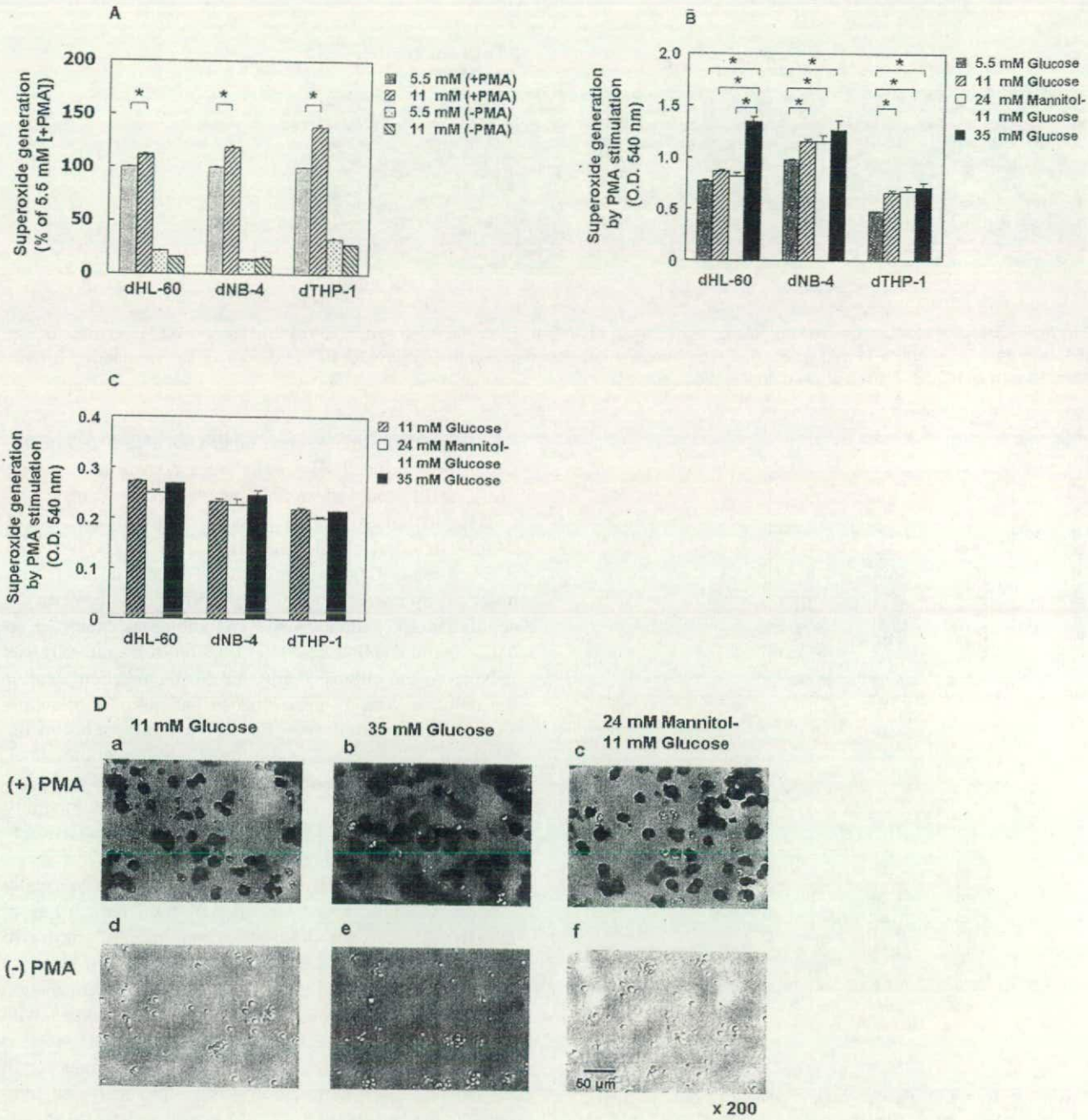

Figure 7. Effect of glucose on the superoxide generation of dHL-60, dNB-4, and dTHP-1 with or without PMA stimulation. The superoxide generation of differentiated and glucose-treated cells was evaluated by NBT reduction assay. Data are mean ± SD from three or more independent experiments. (A) Superoxide generation of the PMA-stimulated or non-stimulated cells after the culture for 24 h with 5.5 mM glucose (shaded bar) and 11 mM glucose (hatched bar). *, $p < 0.0002$. (B) Superoxide generation of the PMA-stimulated cells after the culture for 24 h with 5.5 mM glucose (shaded bar), 11 mM glucose (hatched bar), 24 mM mannitol-11 mM glucose (open bar), and 35 mM glucose (solid black bar). *, $p < 0.0001$. (C) Superoxide generation of the PMA-stimulated cells after the culture for 5 h (short-term) with 11 mM glucose, 24 mM mannitol-11 mM glucose, and 35 mM glucose, no significance was observed. (D) Morphological characteristics of the PMA-stimulated and non-stimulated dHL-60 cells cultured for 24 h with 11 mM glucose (a and d), 35 mM glucose (b and e), 24 mM mannitol-11 mM glucose (c and f). The blue-colored dHL-60 observed under the phase contrast microscope shows the formazan-deposited cells due to superoxide generation after PMA stimulation.

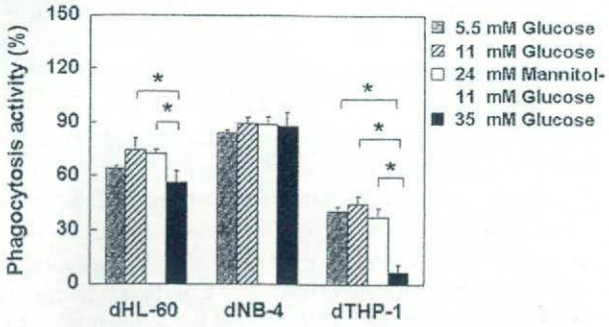

Figure 8. Effect of glucose on the phagocytosis activities of dHL-60, dNB-4, and dTHP-1. After differentiation, the cells were exposed to different concentrations of glucose (5.5 mM, 11 mM or 35 mM) for 24 h. Phagocytosis activity is expressed as the percentage of the cells ingesting. Data are mean ± SD from four independent experiments. *, $p < 0.001$.

The effect of glucose on the cellular phagocytosis was investigated. By culture with 35 mM glucose for 24 h, the phagocytosis of yeast particles by dHL-60 cells significantly decreased compared to the cultures with 11 mM glucose as well as 24 mM mannitol-11 mM glucose (Fig. 8, $p < 0.001$). In dTHP-1 cells incubated with 35 mM glucose for 24 h, phagocytosis activity extremely decreased compared to the cultures with the lower concentration of glucose and 24 mM mannitol-11 mM glucose ($p < 0.001$).

4 Discussion

To our knowledge, this study for the first time demonstrated that exposure of PMN-like differentiated cell lines to higher concentration of glucose (up to 35 mM) caused (i) the enhancement of cellular aggregation in dHL-60, (ii) the reduction of cellular fragility in dHL-60 and dTHP-1, (iii) the enlargements of intracellular vacuoles by PMA stimulation in dNB-4, (iv) the impairments of phagocytosis activities in dHL-60 and dTHP-1, and (v) the induction of superoxide generation by PMA stimulation. Because the culture of differentiated cell lines with 24 mM mannitol-11 mM glucose did not influence the aggregation, fragility or vacuolization, the osmotic effect can be excluded. The other studies showed that GM-CSF, which has a priming effect, stimulates neutrophil aggregation [28], but does not stimulate adherence to endothelial monolayers [29]. In contrast, tumor necrosis factor-α (TNF-α) and lipopolysaccharide, which are triggers, enhance neutrophil adherence to endothelium [30], but do not promote neutrophil aggregation [31]. Our study suggests that the expression of CD11b was not enhanced by high glucose treatment (data not shown). Vedder *et al.* [32] suggest that, as with adherence to endothelium, increased surface expression of CD11b is not the mechanism responsible for aggregation. Taken together, hyperglycemic conditions may play a role similar to GM-CSF in triggering immune responses of ATRA-induced differentiation.

The cytoplasmic vacuolization and/or toxic granulation of neutrophils have been demonstrated more often in bacteremic patients with high NBT reduction capacity [33]. Malcolm *et al.* [34] have reported that no significant differences in the extent of vacuolization were found among healthy donors, toxic patients without bacterial infections, and patients with bacterial infections in the absence of documented bacteremia. The extent of vacuolization was significantly greater in bacteremic patients when compared with the other patients, including patients with bacterial infection without bacteremia [34]. In our study, the effect of high glucose on the vacuolation of neutrophil-like cells by PMA stimulation to mimic bacterial infection was investigated. The dNB-4 cells under high glucose condition with 35 mM were extensively vacuolated by PMA stimulation, suggesting that the high glucose may enhance the ability of vacuolization due to some stimulation in neutrophil-like cells. The patients with type I diabetes mellitus were characterized by a significant impairment of the PMN-mediated phagocytosis [5, 35]. In our study, the phagocytosis activities of dHL-60 and dTHP-1 were significantly impaired in the cultures with high glucose for 24 h, suggesting the association with the pathogenicities in diabetes.

Oliveira *et al.* [36] have indicated that NBT reduction in incubation of rat pancreatic islets as well as neutrophils with 5.6, 8.3, and 16.7 mM of glucose for 1 h increased when compared with the absence of glucose. They further confirmed the involvement of NAD(P)H oxidase activation through protein kinase C (PKC) in the stimulatory effect of glucose by incubation with PMA (a PKC activator), bysindoylmaleimide (GF109203X) (a PKC-specific inhibitor), and diphenylene iodonium (an NAD(P)H oxidase inhibitor) to abolish the increase of NBT reduction induced by glucose. Although we do not know whether the NBT reduction in incubation of neutrophil-like cells with 5.5 mM glucose increases when compared with the absence of glucose, without PMA stimulation no superoxide production of differentiated cells was observed in incubation with any concentration of glucose in this study. Furthermore, the superoxide production by dHL-60, dNB-4, and dTHP-1 cells in incubation with higher glucose (11 or 35 mM) for long-term treatment (24 h) significantly increased when compared with 5.5 mM glucose (physiological concentration), in particular, the NBT reduction of dHL-60 in incubation with 35 mM glucose was extremely induced. However, no significant superoxide production in response to PMA was observed in the short-term treatment (5 h) of dHL-60 treated with 35 mM glucose, suggesting that longer-term treatment with high glucose seems to more enhance the induction of superoxide production. Since without PMA stimulation the NBT reduction of dHL-60 cells cultured with high glucose was not observed, the high glucose condi-

tion probably promotes the priming of neutrophil-like cells rather than the direct triggering.

Osmotic pressures of glucose-free RPMI, 5.5 mM glucose-containing RPMI, 11 mM glucose-containing RPMI, and 35 mM glucose-containing RPMI, which were estimated are approximately 250–290, 255–295, 265–300, and 300–350 mOsm/kg, respectively. In most of tissue culture media that are supplied by manufactures, the osmotic pressure is usually adjusted to the range of 260–320 mOsm/kg to keep isotonic condition. Osmotic pressures of 5.5 mM glucose- and 11 mM glucose-containing media are almost in the range. Only 35 mM glucose-containing medium may give the cell lines a little hypertonic shock. Therefore, we used 24 mM mannitol-11 mM glucose as such hypertonic control. In dHL-60 as shown in Fig. 7B, the ROS induction of 35 mM glucose (a little hypertonic)-treated cells was significantly increased by PMA stimulation when compared with those of the cells treated with 5.5 mM glucose (isotonic) and 11 mM glucose (isotonic), and 24 mM mannitol-11 mM glucose (the same osmotic condition as 35 mM glucose). This suggests that the increase of ROS production is dependent on very high glucose condition. In the cases of dNB-4 and dTHP-1, the ROS productions of 11 mM glucose (isotonic), 24 mM mannitol-11 mM glucose (a little hypertonic), and 35 mM glucose (a little hypertonic) were significantly increased by PMA stimulation when compared with 5.5 mM glucose (isotonic), suggesting that the increase of glucose concentration from 5.5 to 11 mM, but not hypertonic shock, seems to be related to the ROS induction. Thus, dHL-60 appears to become more sensitive by treatment with very high concentration of glucose such as 35 mM on ROS production by PMA stimulation, but dNB-4 and dTHP-1 are likely more sensitive by moderately high concentration (more than 5.5 mM and less than 35 mM) of glucose. The degree of effect may be dependent upon the sensitivity differences of those three cell lines against glucose concentration or treatment time, etc.

The reduction of NBT dye and the generation of ROS are thought to measure indirectly the bactericidal function of neutrophils. There is evidence demonstrating that bactericidal ROS production by activated PMN is reduced in diabetic patients with or without any infections [9] and in diabetic rats [37]. However, there is also evidence that it is normal [38] or increased [39] in patients with type 1 diabetes mellitus. Additionally, Nabi *et al.* and Bellinati-Peies *et al.* have suggested that reduction of NBT by neutrophils is not correlated with their bactericidal activity [35, 40]. They go on to suggest that only the complete absence of NBT reduction reflects low bactericidal activity in neutrophils. Based on those reports, a question is how we can explain exactly what the present findings of glucose-induced ROS production means in relation to bactericidal function of neutrophils in patients with type 1 diabetes. One of explanations is that the type 1 diabetes is much more complicated, because the patient blood with the diabetes included increased glycated proteins due to Maillard reaction, *e.g.* AGEs, as well as high glucose. Wong *et al.* [41] showed that AGEs stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A2 and generation of arachidonic acid. Moreover, De Toni *et al.* [42] have represented that in patients with diabetes the impact on PMN function is of multifactorial origin, and is probably correlated to the glucose level and to glycation of PMN protein, such as NADPH oxidase or myeloperoxidase. Alternatively, glucose in PMN may be reduced by aldose reductase to polyols, and this pathway requires NADPH, the coenzyme for the respiratory burst. They further found that superoxide production in response to opsonized zymosan was reduced in diabetic patients. The activation of protein tyrosine kinase is an important mechanism underlying transmembrane signaling and, the protein tyrosine phosphorylations, stimulated by zymosan receptor-mediated activation, might be caused by the activation of specific protein tyrosine kinase, whereas activation by PMA is probably mediated through another PKC type. In our study, therefore, we focused on the effect of high glucose alone (not including AGEs etc.) in ROS production at the mimicked initial step of maturated neutrophils released from bone marrow to blood using three differentiated cell lines.

Taken together, in response to glucose, HL-60 cells out of three cell lines seem to be better as a neutrophil model, but NB-4 and THP-1 (as well as HL-60) are still expected to have the potential responses similar to *in vivo* neutrophils under the consideration of sensitivity difference against glucose among those cell lines. By this possibility, we think that the three cell lines (HL-60, NB-4, and THP-1) are probably useful for better understanding of neutrophil function as a model *in vitro* experiment for long-term treatments (24 h or longer). Thus, this study provides significant and basic information regarding the effect of high glucose on the functional abnormalities of neutrophil-like differentiated cell lines.

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The authors have declared no conflict of interest.

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Genotoxicity and Estrogenic Activity of 3,3′-Dinitrobisphenol A in Goldfish

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3,3′-Dinitrobisphenol A (dinitro-BPA) is formed in a mixture of bisphenol A (BPA) and nitrite under acidic conditions. It shows genotoxicity in male ICR mice on a micronucleus test, but its estrogenic activity has not been examined *in vivo*. We examined its estrogenic activity using goldfish (*Carassius auratus*) by measuring plasma levels of vitellogenin (VTG) by the ELISA method. Expression of VTG didn't increase in the plasma of goldfish intraperitoneal injected with dinitro-BPA at a dose of 10 mg/kg of body weight.

We also examined the genotoxicity of dinitro-BPA by single-cell gel electrophoresis (comet assay) and a micronucleus test using goldfish. The DNA tail moment of blood cells increased after intraperitoneal injection of dinitro-BPA. Dinitro-BPA at the same dose significantly increased micronucleus frequency in gills of goldfish. On the other hand, BPA did not significantly increase the frequency of micronucleated cells.

In conclusion, we found that dinitro-BPA did not show estrogenic activity, but had genotoxic potency stronger than that of BPA.

Key words: goldfish; 3,3′-dinitrobisphenol A; vitellogenin; comet assay; micronucleus test

It is known that various chemicals in our environment show substantial influences on aquatic animals and mammals, including humans. Among these compounds, bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA), which is used as an ingredient in the manufacture of epoxy carbonate, polycarbonate and polyester styrene, exhibits estrogenic activity. The estrogenic activity of BPA has been demonstrated using various assays. Krishnan *et al.* found that BPA showed estrogenic activity in a culture assay using MCF-7 human breast cancer cells.[1)] Hashimoto *et al.* revealed estrogenic activity of BPA using the yeast two-hybrid system.[2)] It has been reported that BPA bound with human estrogen receptor alpha, beta, and gamma.[3–5)] Hence it is said to be an endocrine-disrupting chemical (EDC). It is also used in food packaging and can-coating agents and in dental sealants, and is readily orally-ingested by humans. Consequently, it is important to examine the toxicity of BPA in human body after oral intake.

BPA has not been recognized as a mutagen by several *in vitro* and *in vivo* mutagenicity assays. Haworth *et al.* reported that it showed negative results in bacterial reverse mutation tests using *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537.[6)] Ivett *et al.* reported that it did not exhibit mutagenic activity in an *in vitro* genotoxicity test with Chinese hamster ovary cells.[7)] Gudi *et al.* also reported that it did not increase the frequency of micronucleated reticulocytes (MNRETs) in bone marrow of mice.[8)] Recently, however, there have been positive results to the effects that it shows mutagenic activity. Hilliard *et al.* reported that BPA exhibited positive effects in an *in vitro* chromosome aberration test using CHO cells without S9 mix.[9)] Tayama *et al.* demonstrated that it induced genotoxicity in CHO-K1 cells using sister-chromatid exchange and comet assay.[10)] Tsutsui *et al.* found that quinone compounds formed from BPA formed DNA adducts in an *in vitro* 32P-postlabeling assay.[11)] Thus BPA shows opposite effects on mutagenic activity in different test systems.

Humans regularly consume nitrite and nitrate through vegetables and tap water, and in their daily diet as food additives.[12)] Nitrate is readily reduced to nitrite by oral bacteria. Some scientists have reported that mutagenic/carcinogenic nitrosamines are formed by the reaction of nitrite and secondary amines in foodstuffs under acidic conditions.[13)] Several phenolic compounds also show mutagenic activity after nitrite treatment. Wakabayashi *et al.* found that some phenol and indole derivatives present in the environment are changed to mutagenic compounds by nitrosation.[14)] Kikugawa and Kato found

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that diazoquinone compounds, which showed strong mutagenicity, were formed by interaction between phenol and nitrite.[15,16] We have found that BPA showed mutagenic activity on treatment with nitrite under acidic conditions, and dinitro-BPA, which induced micronuclei in peripheral erythrocytes of ICR male mice, was formed in a reaction mixture of BPA and nitrite.[17] Additionally, we found that dinitro-BPA did not show estrogenic activity on *in vitro* assay. However, we have not yet determined whether dinitro-BPA shows estrogenic activity on an *in vivo* assay.

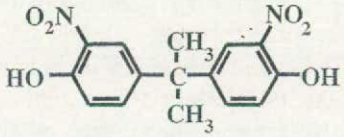
Some scientists have examined the estrogenic activity of endocrine disruptors by *in vivo* tests using aquatic organisms. Warner *et al.* demonstrated the estrogenic activity of BPA using fathead minnow.[18] Tabata *et al.* used Japanese medaka (*Oryzias latipes*) to confirm the endocrine disrupting action of BPA.[19] Ishibashi *et al.* also confirmed the estrogenic activity of nonylphenol by detecting the vitellogenin concentration in plasma of goldfish.[20] Goldfish can be bred easily and cheaply and are available all over the world. We have also examined the genotoxicity of various chemicals in river water using goldfish. Judging by these reports, goldfish is a useful organism in investigating the mutagenic and estrogenic activities of various chemicals.

In the present study, we examined the mutagenic and estrogenic activities of BPA and dinitro-BPA with goldfish.

Materials and Methods

Chemicals. BPA, 17β-estradiol (E2), acridine orange, methyl metahnesulfonate (MMS), and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemicals (Osaka, Japan). 3,3′-Dinitro-bisphenol A (dinitro-BPA) was synthesized as described by Masuda *et al.*[17] Figure 1 shows the chemical structure of dinitro-BPA. Low melting point (LMP) agarose and normal melting point (NMP) agarose were from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY). Mitomycin C (MMC) and ethidium bromide were from Kyowa Hakko Kogyo (Tokyo) and Merck (Darmstadt, Germany) respectively.

Fish. We obtained goldfish weighing 11 ± 2 g from a local dealer in Hamamatsu, Japan. Before the experiment, goldfish were acclimatized for 2 weeks in a well-aerated aquarium at 18 ± 2 °C.



3,3'-dinitrobisphenol A (dinitro-BPA)

Fig. 1. Chemical Structure of Dinitro-BPA.

Treatment of fish. In a previous study, we injected chemicals from a river intraperitoneally to examine their mutagenic activities.[21] In a similar way, BPA and dinitro-BPA were dissolved in DMSO and injected intraperitoneally once at 1 and 10 mg/kg of body weight. Three to 5 fish were used in each group. In the negative control group, DMSO was injected intraperitoneally in place of the test chemical. In the positive control group, we injected intraperitoneally E2 (1.0 mg/kg of body weight), MMS (50 mg/kg of body weight) and MMC (4.0 mg/kg of body weight) for VTG determination and in comet assay and micronucleus test. For determination of VTG in plasma, at 96 h after injection blood was collected and centrifuged at 3,000 rpm for 20 min, and the plasma was divided into aliquots and stored at −20 °C until use. In the comet assay, 3 h after injection of chemicals, peripheral blood was collected. Peripheral blood and gills were obtained at 96 h after injection of chemicals in the micronucleus test.

Vitellogenin analysis. Several researchers have found that the VTG molecule structure of carp (*Cyprinus carpio*) is similar to that of other cyprinid fish. Zhong *et al.* determined VTG production of hepatocytes in a rare minnow (*Gobiocypris rarus*) by ELISA using an antibody against carp (*Cyprinus carpio*) VTG.[22] This antibody has been found to bind to VTG produced by other cyprinid species, such as goldfish and fathead minnow.[23] Hence the carp VTG antibody is used to measure VTG concentration in plasma of a wide variety of cyprinid fish, such as goldfish. In this study, a Carp VTG ELISA Kit (Trans Genic, Japan) was used to determine VTG contents in plasma of goldfish according to the manufacturer's instructions. The concentrations of VTG were calculated from the linear part of the log-transformed VTG standard curve. The detection limit of VTG was 0.04 µg/ml plasma.

Alkaline comet assay. The alkaline comet assay was performed according to the method of Tice *et al.*, with some modifications.[24] Five µl of blood was diluted with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS(-): 7.5 g of NaCl, 0.2 g of KCl, and 0.2 g of sodium bicarbonate dissolved in 1 liter of water), and samples were mixed with 75 µl of 1% low melting point (LMP) agarose. The mixture (75 µl) was layered on a 1% LMP agarose layer and covered with 75 µl of 1% LMP agarose. After preparation, the slide was immersed in lysing solution and refrigerated at 4 °C for 1 h. After lysis, the slide was placed in alkaline electrophoresis buffer for 10 min to allow salt equilibration and further DNA unwinding. Electrophoresis was performed at 30 V, 300 mA for 15 min at 4 °C. The slide was then neutralized with 0.4 M Tris buffer (pH 7.5) for 10 min. The cells were stained with 50 µl of ethidium bromide (20 µg/ml). Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. One hundred cells were examined

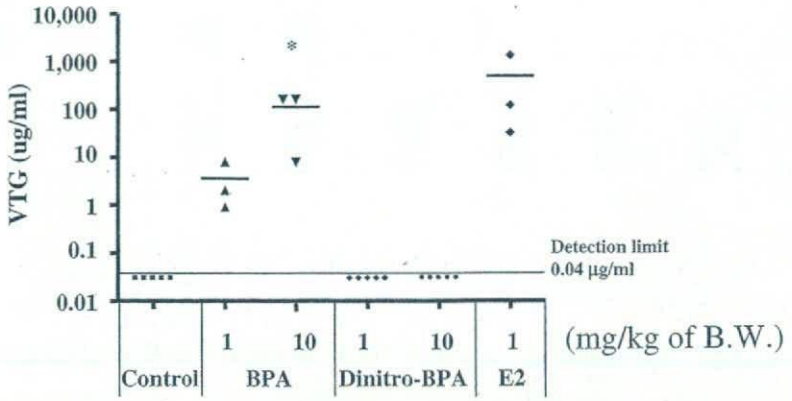


Fig. 2. VTG Concentration of Plasma in Goldfish by Injection of BPA and of Dinitro-BPA.
Mean values were obtained from three to five fish. The bars represent SD values. *$p < 0.01$ (*vs.* control).

per fish. The tail moment of DNA was measured using Comet Analyzer Youworks Bio Imaging Software.

Micronucleus test. The micronucleus test was performed according to the methods of Ueda *et al.*,[25] and Hayashi *et al.*[26] with some modifications. Peripheral blood of goldfish was collected from a caudal vein using a heparinized syringe. Ten µl of peripheral blood was diluted with 40 µl of fetal bovine serum. Nine µl of diluted blood was spread on an acridine orange-coated glass slide. Three gills on each side of the were excised and washed with PBS(-). Gills were transferred to 5 ml of PBS(-) and broken up with forceps. Tissue clumps and gill arches were removed and discarded. Free cells were collected by centrifugation (1,000 rpm, 5 min) and treated with 2 ml of 75 mM KCl hypotonic solution for 5 min. Then 0.1 ml of Carnoy fixative A (acetic acid and methanol, 1:3 by vol.) was added and centrifuged at 1,000 rpm for 5 min. A half volume of the supernatant with cells was suspended in 5 ml of Carnoy fixative B (acetic acid and methanol, 1:99 by vol.) and centrifuged. Then three fourth of the supernatant was removed and the cell suspension was dropped on a slide glass smeared with 0.0025% acridine orange solution. At least 1,000 erythrocytes and gill cells were observed with a fluorescence microscope (magnification, 400×) and the numbers of micronucleated cells were recorded.

Statistical analysis. Dunnett's test after one-way ANOVA was used to evaluate the significance of differences in VTG contents, tail moment in the comet assay, and micronucleus frequency in the micronucleus test between fish treated with BPA and with dinitro-BPA and the untreated group, A p-value lower than 0.05 was considered to be statistically significant.

Results and Discussion

VTG a phospholipoglycoprotein precursor of egg yolk protein, is synthesized in the liver of sexually mature oviparous females.[27] It is normally undetectable in the plasma of males and immature females. However, when male fish were treated with EDCs, VTG was detectable in their plasma. Hence VTG in plasma of male and juvenile fish treated with chemicals is used as a biomarker in evaluation of estrogenic activity of chemicals.[28]

In this study, we examined the estrogenic activities of BPA and dinitro-BPA by quantifying of VTG by the ELISA method with carp VTG antibody. The VTG concentration (113.5 µg/ml) in plasma of goldfish was significantly increased by treatment with BPA (10 mg/kg of body weight) ($p < 0.01$) (Fig. 2), but dinitro-BPA did not show any significant expression of VTG protein in plasma (n.d.). We have also found that the estrogen (α) binding potency of dinitro-BPA was weaker than that of non-treated BPA by *in vitro* assay.[17] In addition, we have reported that the estrogenic activity of 2-nitro-17β-estradiol, which is formed in a reaction mixture of 17β-estradiol and nitrite, was weaker than that of E2 using an ELISA kit.[29] Hence it is possible that binding potency to the estrogen receptor changed because the physico-chemical properties of BPA were transformed by nitration.

Comet assay is a rapid and sensitive method of detecting DNA single-strand breaks and alkali-labile sites in individual cells. Since these types of DNA damage can be the initial damage induced by genotoxic chemicals, comet assay is generally used as a method of detecting DNA damage due to exposure with mutagens and carcinogens.[30] Figure 3 shows the mean values of DNA tail moment in peripheral erythrocytes induced by dinitro-BPA and BPA. The mean tail moment values were increased by intraperitoneal injection of BPA (5.06 ± 0.37) and dinitro-BPA (8.20 ± 0.67) at 10 mg/kg of body weight. Variance analysis ($p < 0.01$) of these values showed a significant difference in DNA damage as between the dinito-BPA treated group and BPA-treated group.

Figures 4 and 5 show the frequencies of micronuclei in peripheral erythrocytes and gills of goldfish respectively.

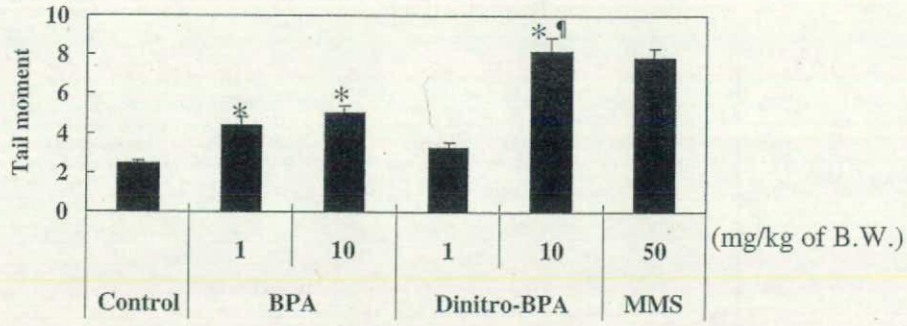


Fig. 3. Tail Moment of Peripheral Erythrocytes in Goldfish Due to Injection of BPA and of Dinitro-BPA.
One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represent S.E.M. values.
*$p < 0.01$ (*vs.* control), ¶$p < 0.01$ (*vs.* 10 mg/kg of B.W. BPA).

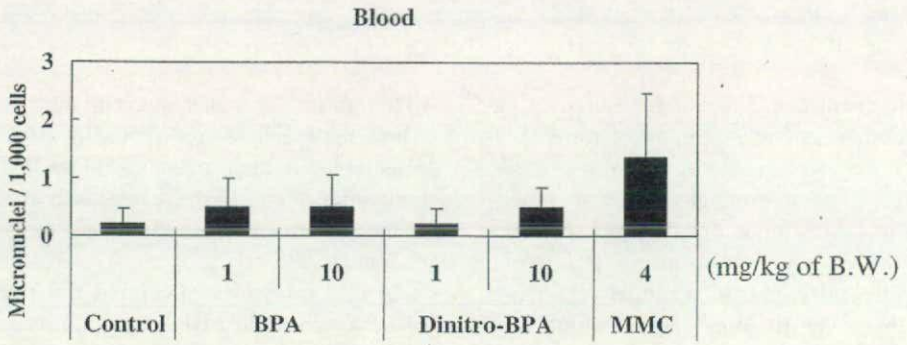


Fig. 4. Change in Frequency of Micronuclei in Peripheral Erythrocytes of Goldfish Injected with BPA and with Dinitro-BPA.
Mean values were obtained from five fish. The bars represent S.D. values.

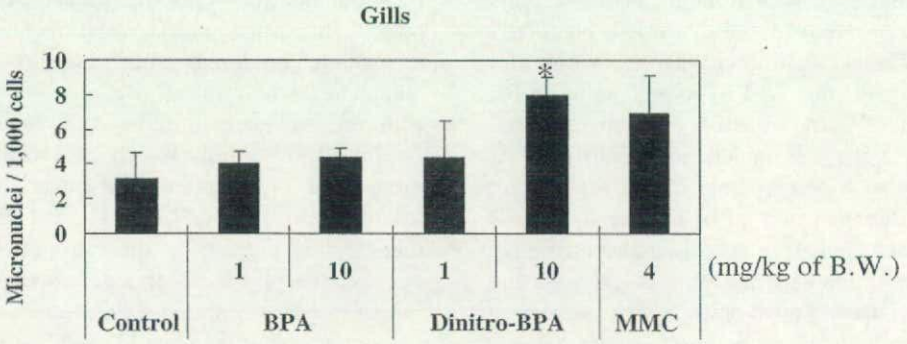


Fig. 5. Change in Frequency of Micronuclei in Gills of Goldfish Injected with BPA and with Dinitro-BPA.
Mean values were obtained from five fish. The bars represent S.D. values. *$p < 0.01$ (*vs.* control).

The frequency of micronuclei in gills increased significantly after intraperitoneal injection of dinitro-BPA at a dose of 10 mg/kg of body weight (8.00 ± 1.00) as compared with the control group ($p < 0.01$), but dinitro-BPA did not induce micronuclei in peripheral erythrocytes. Hayashi *et al.* have reported that mutagen-treated fish showed higher frequencies of micronucleated cells in gills than in peripheral erythrocytes, and recommended the use of gill cells in fish micronucleus assays.[26] BPA did not induce micronuclei in either gills or erythrocytes. Michael *et al.* have reported that BPA caused DNA damage due to apoptosis induced by BPA.[31] In this study, BPA showed DNA-damaging potency on the comet assay, but did not induce micronuclei on the micronucleus test at significant frequency. On the other hand, dinitro-BPA showed DNA damaging potency and induced micronuclei. These results suggest that the toxicity of BPA is different from that of dinito-BPA. Therefore, dinitro-BPA is assumed to induce abnormal chromosomes and to act as a genotoxic chemical.

The genotoxic effects of nitro compounds are generally linked to nitrate reductase. There are several kinds of NADPH-cytochrome *c* reductase,[32] xanthine oxidase,

DT-diaphorase,[33,34] and other enzymes. Nitro compounds show genotoxic activity through two pathways. The first reduction step is the formation of the nitrogen radical anion. Under aerobic conditions, this radical is reoxidized by O_2 and produces superoxide and hydroxyl radical anions. Under anaerobic conditions, reductive reaction leads to the formation of nitroso and hydroxylamine derivatives. Hydroxyl radicals cause DNA strand breaks.[35] Hydroxyl amino groups induce the transformation of DNA adducts.[36] These metabolic responses can cause DNA damage and abnormal chromosomes *in vivo*. Hence it is necessary to measure nitrogen reductase activity in goldfish.

Recently, many problems of nitrogen pollution have appeared in the water environment,[37] in acid rain,[38] and in the acidification of freshwater.[39] When these environmental conditions occur simultaneously, a nitro compound might be generated in the environment. Telscher *et al.* reported the formation of a nitrogen metabolite of nonylphenol isomer in soil/sewage sludge mixtures.[40] In the water phase, nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) can be formed by a photochemical reaction of PAH, with nitrite donating the nitro group.[41,42] There are various nitro compounds in the urban air and soil.[43–45]

The present study indicates that the estrogenic activity of dinitro-BPA was lower than that of BPA in goldfish. However, the genotoxic potency of dinitro-BPA was stronger than that of BPA. We also found that E2 and nitro-E2 showed similar behaviors.[29] Other nitro compounds might be formed and flow into the water environment. As a result, it is possible that aquatic organisms and human beings are exposed to nitro compounds. Hence we must investigate the formation of nitro compounds and evaluate their estrogenic and genotoxic activities *in vivo*. In addition, we must monitor dinitro-BPA and other nitro compounds in the environment.

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Review Article J. Clin. Biochem. Nutr., 43 Suppl. 1, 22–23, July 2008

Effect of Tea Extracts on Gastric Mucosal Erosion and Hemorrhage in *Helicobacter pylori* Infected Mongolian Gerbils

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Summary **In this study, we examined the inhibitory effect of green tea extract and catechins on the growth of *Helicobacter pylori* (*H. pylori*) and also on the improvement efficiency in *H. pylori* infected Mongolian gerbils. The possibility of eradication of *H. pylori* by oral administration of the catechins was also investigated. By administration of catechin adsorbed sucralfate for 10 days to *H. pylori* infected Mongolian gerbils, the colony forming units of *H. pylori* was significantly decreased. The combined effect of catechins and omeprazole, a proton pump inhibitor, was observed in an increase of pH as well as decrease of the mucosal hemorrhage. These results are supported by the epidemiological studies in green tea producing and non-producing area.**

***Key Words*: *H. pylori*, green tea, catechin, gastric ulcer**

The correlation between *Helicobacter pylori* (*H. pylori*) infection and gastric diseases, such as chronic gastritis, peptic ulcers, intestinal metaplasia and gastric cancer, has been widely investigated. In 1994, the World Health Organization/International Agency for Research on Cancer (WHO/IARC) concluded that *H. pylori* is one of the definite carcinogens based on the epidemiological findings. An epidemiological study shows that half numbers of Japanese are infected with *H. pylori*.

In a clinical field, the eradication is now performed by combined administration of amoxicillin, clarithromycin of antibiotics and lansoprazole of proton pump inhibitor. In recent year, side effects and resistance to bacteria are often observed after the administration of these medicines. Therefore, usage of natural products has been desired for the eradication of *H. pylori* infection diseases. Several investigators demonstrated the suppression effects of daily food stuffs containing Wasabi on *H. pylori*-induced gastric in Mongolian gerbils (M. Iimuro *et al.*, 2002, S. Matsubara

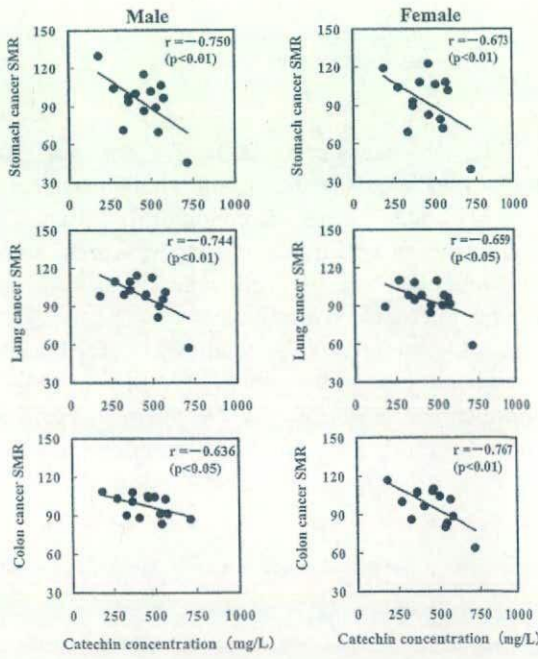

Fig. 1. Relationship between catechin concentration in the brewed green tea and cancer SMR in 14 areas (1995–1999)

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Table 1. Combined effect of green tea catechins and anti-*H. pylori* agents

a) Combined effect of green tea catechins and anti-*H. pylori* agents

Test systems	MBC (μg/ml)	
	NCTC	YS50
AMPC + GTC (1.25 mg/ml)	0.186	0.047
AMPC	0.186	0.186
CAM + GTC (1.25 mg/ml)	0.8	0.8
CAM	6.4	6.4

MBC: Minimal Bactericidal Concentration

GTC: Polyphenon 70S (Tokyo Food Techno, Tokyo Japan) contains 73.4% catechins (EGC: 33.5%, ECG: 10.1%, EGC 17.4%, EC: 8.6%)

b) Combined effect of green tea catechins and sucralfate in *H. pylori* infected mongolian gerbils

Experimental group (No. of animals)	GTC administration (mg daily)	Intragastric pH[a]	Bacterial count (log CFU/stomach)[b]
I Control (4)	0	2.7 ± 0.9	5.690 ± 0.268
II Sucralfate (4)	0	2.8 ± 0.5	5.655 ± 0.071
III GTC solution (4)[c]	20	3.3 ± 0.3	5.353 ± 0.197
IV GTC-Sucralfate (6)[d]	5	2.6 ± 0.4	5.538 ± 0.197
V GTC-Sucralfate (6)[d]	10	2.8 ± 0.7	5.088 ± 0.339
VI GTC-Sucralfate (6)[d]	20	2.8 ± 0.4	3.839 ± 0.476*

*Significantly reduced compared with the Control group, at $p<0.05$ Sucralfate: Anti-gastritis for gastric membrene erosion GTC, green tea catechin; Polyphenon 70S

[a] The average pH of the stomach contents. The values for results are expressed as means ± SDs. Bonferroni's test was used for statistical analysis.

[b] I The average bacterial count each group was calculated. The values for results are expressed as means ± SDs. Bonferroni's test was used for stastistical analysis

[c] The contcentration of GTC solution was 20 mg Polyphenon 70S/ml distilled water. The animals were orally administered 1 ml of the solution daily for 10 days

[d] The contcentration of GTC-Sucralfate was 20 mg Polyphenon 70S/ml Ulecermin suspension. The animals were administered 0.25 ml (group IV), 0.5 ml (group V), and 1 ml (group VI) of GTC-Sucralfate daily for 10 days

et al., 2003, H. Masuda *et al.*, 2004, T.Y. Oh *et al.*, 2005). Recently, it was found that tea catechins inhibited the growth of *H. pylori* under the neutral condition (K. Mabe *et al.*, 1999). *H. pylori* was eradicated in 10 to 36% of the catechin treated Mongolian gerbils with significant decreases in mucosal hemorrhage and erosion.

In this study, we present the inhibitory effect of green tea extract and catechins on the growth of *H. pylori* and also on the improvement efficiency in *H. pylori* infected with Mongolian gerbils. The possibility of eradication of *H. pylori* by the oral administration of the catechin was also investigated. By Oral administration of catechin adsorbed sucralfate for 10 days to Mongolian gerbils infected with *H. pylori*, the colony forming units of *H. pylori* was significantly decreased. The combined effect of catechins and omeprazole, was demonstrated by the increase of pH as well as decrease of the mucosal hemorrhage. These results are supported by the epidemiological data in tea producing and non-producing areas.

総　説

沢わさびの機能性

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Functional Properties of Sawa-Wasabi (*Wasabia japonica*)

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Sawa-wasabi (*Wasabia japonica* Matsumura) is a traditional spice and a pungent food in Japan, and contains various components, such as isothiocyamates, vitamin C and polyphenols in roots, stems and leaves. They are known to exert some functional properties to bacteria and mammals including humans.

Wasabi extracts show antibacterial activity to some pathogens. The growth of fungus on foods is inhibited by wasabi stems, too. The activities are attributed to several isothiocyanates, such as allyl and 6-methylsulfinylhexyl isothiocyanates, and expressed by the binding potency of isothiocyanates to peptides or proteins in cell membrane and cytoplasm of wasabi. Wasabi also shows bactericidal activity to *Helocobacter pylori*. The extract shows inhibitory effect on urease activity of *H.pylori*. Gastric mucosa injury of *H.pylori*-infected *Mongolian gerbil* was suppressed by administration of wasabi samples. Furthermore, the combined inhibitory effect between wasabi extracts and medicines on gastric mucosa injury was confirmed. Wasabi extracts also inhibited gastric mucosa injury in stomach of rats induced by shackled stress.

Wasabi also has radical scavenging activity and inhibitory effect on lipid peroxidation in vitro. In vivo test, oral administration of wasabi samples to ICR mice inhibited oxidative abnormal chromosome induced by gamma-ray irradiation. Wasabi contained vitamin C and antioxidative polyphenols, such as kaempferol, myricitrin, luteolin.

Wasabi was also found to inhibit mutagenic activity of 2-amino-3,8-dimethlimidazo[4,5-*f*]-quinoxaline (MeIQx) or 3-chloro-4-dichlomethyl-5-hydroxy-2(5*H*)-furanone(MX), and compounds containing ally isothiocyanate were identificated as active components. Isothiocyanates strongly restrained the cancer cell proliferation. It is thought that isothiocyanates might show various potencies, such as, detoxification of carcinogens, modification or deletion of cancer cells and immunostimulatory activity.

Moreover, wasabi shows inhibitory effect on Maillard reaction, orexigenic effect, enhancing action on vitamin B_1 synthesis, inhibitory effect on platelet aggregation, enhancing of gastric absorption, the effect on secretory property of colon electrolyte, antiallergic action.

Therefore, wasabi may be positioned as various functional foods, and the development of new food products with wasabi is promising.

近年、食生活を通して健康の維持や増進をはかることを目的として、食品素材の機能性に関する研究が積極的に行われている。日本の伝統食品素材である沢わさびの機能性については、古くは飛鳥京苑池遺構の木簡に「委佐俾三升」の記述が残されており、江戸時代には貝原益軒や小野蘭山をはじめとする多くの医家、漢学者が著書の中でわさびの効能を取り上げている。また、最近我々を含めて沢わさびの機能性に関する総説が出版されてい

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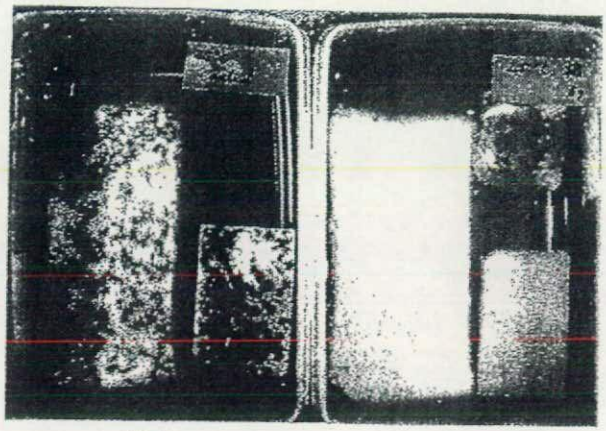

対照　　わさび添加

食パン(密閉容器中保存。25℃、8日後)

Fig. 1　わさびの抗カビ効果

る。ここではそこで、沢わさびの有する各種機能性に関して、最近の知見や著者らの研究成果を含めて述べる。

1．抗菌作用と抗カビ作用

沢わさびの抗菌作用に関しては、1932年にオットーショーブルはわさびの液汁がコレラ菌、チフス菌、大腸菌、連鎖菌、肺炎菌、ジフテリア菌、結核菌に対して、殺菌効果を示すことを明らかにした。また、鉄本らは大腸菌、黄色ブドウ球菌、チフス菌、コレラ菌に対する沢わさびの抗菌作用を確認している[1]。我々は、米飯、パン、餅を別々の容器に入れ、密閉室温に保存して、カビの発生状況を観察したところ、それぞれ5、2及び6日後にカビの発生を確認した。しかし、すりおろした沢わさびの根茎を密閉容器内に共存させると、90日を経過してもカビが発生しないことを見い出した（図1）。沢わさびの抗菌作用、抗カビ作用は沢わさびに含まれている辛味成分であるイソチオシアネート（ITC）類が関与していると考えられている。それらITCの中でもわさび中の含有量が最も多いアリルイソチオシアネート（AITC）の抗菌性に関して、結核菌の発見者であるコッホ（1882年）が報告している。AITCは当初から、沢わさび中には存在しておらず、わさびをすりおろすという操作により生成する。ITCは沢わさび中では前駆体であるグルコシノレート（カラシ油配糖体）のシニグリンとして含まれており、すりおろすことにより細胞が破壊されて、酵素のミロシナーゼが作用し、ITCに変換される（Fig. 2）。このように、わさび植物体の中では、シニグリンとミロシナーゼは別々に存在している。これらイソチオシアネートの発生は害虫や病原体に対する植物の生体防御機構と考えられている。沢わさびの他、西洋わさびや中国わさびにおいてもAITCの含有量が最も高く、沢わさびにおいてはω-メチルチオアルキルイソチオシアネート（ω-MTITC）とω-メチルスルフィニルアルキルイソチオシアネート（ω-MSITC）も比較的多く含まれている。しかし、西洋わさび（わさび大根）にはこれらの含有量が少なく、β-フェネチルイソチオシアネートが多い。中国わさびには末端に二重結合を有するアルケニルイソチオシアネートが多く含まれている（Table 1）[2]。

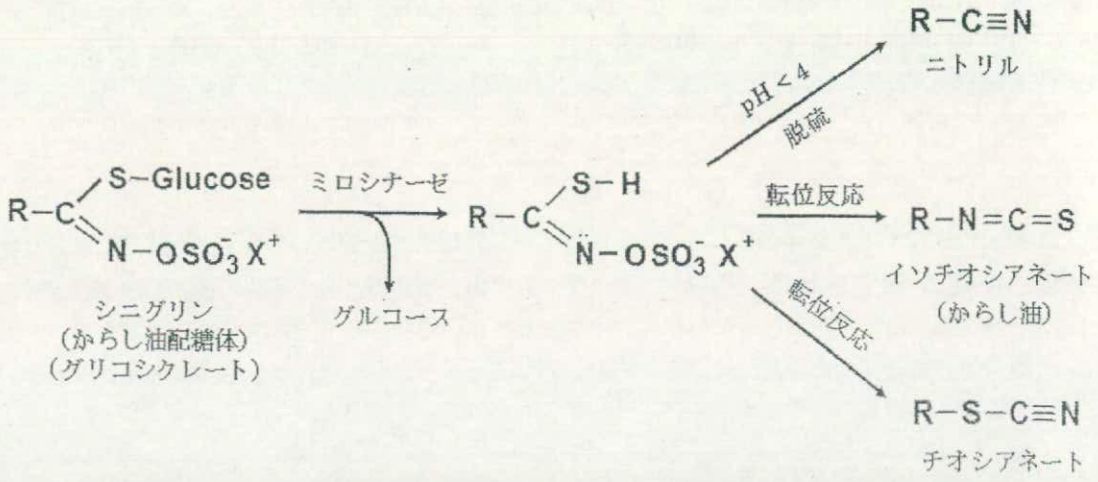

Fig. 2　イソチオシアネートの生成機構

Table 1　わさびに含まれるイソチオシアネートの相対含有量（衛藤ら、1990）

からし油	沢ワサビ			西洋ワサビ	中国ワサビ
	根茎	茎	葉	根	根
アリル	370	62	76	322	230
n-ブチル	5.8	1	1.2	1.4	-
3-ブテニル	6.1	0.2	0.9	2.7	24
4-ペンテニル	13	2.2	2.6	3.2	74
5-ヘキセニル	3.4	1	1.9	0.6	51
β-フェネチル	-	-	-	75	200
5-メチルチオペンチル（5-MTITC）	1.6	0.9	0.4	-	
6-メチルチオヘキシル（6-MTITC）	6.3	8.8	3.8	-	trace
7-メチルチオヘプチル（7-MTITC）	4.8	2.0	1.1	-	2.9
5-メチルスルフィニルペンチル（5-MSITC）	6.6	1.0	1.4	2.7	
6-メチルスルフィニルヘキシル（6-MSITC）	26	8.4	18	3.0	
7-メチルスルフィニルヘプチル（7-MSITC）	4.7	1.5	3.6	2.6	36

AITCは溶液よりも気体状態で菌に接触することで強く生育を阻害し[3,4]、酵母、カビ等の真菌類に対しても抗微生物活性が強く、細菌においてはグラム陽性菌よりもグラム陰性菌に対して抗菌効果が強い。また、腸管出血性大腸菌O-157に対しても有効であり、各濃度における増殖曲線は、濃度依存的に増殖が遅延しており、0.05%以上では死滅したと報告されている[5]。また、寒天培地に塗布したO-157と腸炎ビブリオをAITCに0～8時間暴露したところ、曝露時間が長くなるにつれて、生菌数（コロニー数）が顕著に減少した[5]。我々はすりおろした沢わさび根茎や市販のチューブ入りわさび、からしも抗菌作用を有することを確認している。長谷川らはわさびまたはAITCを脂肪が少ない赤身と脂肪の多いツナの懸濁液に加えて、腸炎ビブリオを培養し、経時的に生菌数を測定したところ、脂肪含量の多いツナではより殺菌効果が強く、菌の増殖が抑制されることを報告している[6]。

沢わさび中にはAITC以外の抗菌成分として、他のITCを含有している。6-メチルスルフィニルヘキシルイソチオシアネート（6-MSITC）は沢わさび根茎に含有されている特徴的な成分であり、我々はその類縁体を合成して、腸炎ビブリオに対する抗菌活性を調べた。6-MSITCは強い抗菌性を示し、スルホキシド、スルホンに比べ、スルフィドが強い活性を示すことを明らかにした[7]。さらに、小野らはω-メチルスルフィニルアルキルイソチオシアネートの大腸菌と黄色ブドウ球菌に対する抗菌活性を報告している[8]。チオニン（thionins）もまた、わさびに含まれる抗菌成分である。チオニンはシステインに富んだ低分子量の塩基性タンパク質であり、西原らはわさびの茎葉より2種類のγ-チオニン相同性遺伝子を単離している[9]。

わさびの抗菌、抗カビ活性の作用機構としては、ITCが細胞質や細胞膜のペプチドやタンパク質のOH、NH_2、SH基と反応し、これらを修飾して酵素活性阻害を起こすものと考えられている。特にTCAサイクルに関与する脱水素酵素活性を阻害して解糖系や呼吸系などのエネルギー代謝を阻害すると推定している。浜島らはAITCがアニサキスに対して乳酸生成量や酸素吸収量を抑制すること[10]、また小嶋らは酵母においては酸素吸収が阻害されることを観察しており、それ故、AITCが解糖系とチトクロームCオキシダーゼに対して阻害作用を発現するものと推定している[11,12]。Linらは腸管出血性大腸菌O-157を含む3種の細菌に対し、増殖期と増殖停止期（定常期）におけるAITCへの感受性や培養液中へ漏出してきた細胞膜内の代謝物や酵素活性を調べた[13]。その結果、AITCは増殖期と定常期のいずれにおいても感受性を示し、また菌の代謝物が細胞膜から漏出し、菌数の減少に伴って代謝物やβ-ガラクトシダーゼの漏出を起こした。したがって、分子内に疎水性のアリル基と極性基のイソチオシアネート基を持つAITCは細菌の細胞膜に作用して、細胞内の代謝物の漏出を誘導して殺菌作用を発現しているものと考えられた。また、AITCは昆虫に対しても有効であることからAITCを含有するポリマーが包装用資材として開発されている。

Table 2 ピロリ菌に対する沢わさび抽出物とアリルからし油の抗菌活性

ピロリ菌	アリルからし油 (mg/ml)	沢わさび (mg 乾燥重量 /ml)		
		根茎	葉	茎
NCTC 11637	2.00	156.3	156.3	312.5
		<0.184>	<0.058>	<0.128>
YS 27	4.00	625.0	625.0	625.0
		<0.736>	<0.232>	<0.256>
YS 50	1.00	156.3	156.3	312.5
		<0.184>	<0.058>	<0.128>

値は最小殺菌濃度を示している。
< >の値はアリルからし油の含有量を示している。
NCTC 11637: 標準菌、YS 27: 十二指腸潰瘍由来、YS 50: 胃がん由来

Worfel らは、高分子ポリマーシートに AITC を吸着させた包装材について、貯蔵穀物害虫に対する効果を試験し、タバコシバンムシ (*Lasioderma serricorne*) とヒラタコクヌストモドキ (*Tribolium confusium*) に殺卵性を示したことを報告している[14]。さらにわさび成分は貝類への忌避作用も示す。船底や魚網などに付着するフジツボや藻類を防除するため、これまで銅やスズ化合物が使用されてきたが、環境汚染が問題となった。そこで、伊奈らはムラサキイガイに対するわさび成分の忌避効果を検定し、わさび根茎抽出物を塗布すると、ムラサキイガイの足糸はそのエリアを避けて延伸することを確認した。わさび含有成分であるω-メチルチオアルキルイソチオシアネートとフェネチルイソチオシアネートが特に強い忌避効果を示した[15]。

2. 抗ピロリ菌作用

最近の研究により、胃潰瘍、十二指腸潰瘍、慢性胃炎などの発症にピロリ菌 (*Helicobacter pylori*) 感染が深く関与していることが報告され、胃がん発生の要因の一つとして考えられている。1994年、国際がん研究機関 (IARC) ではピロリ菌がヒトに対して胃がんを発生しうる重要なリスクファクターと位置づけている。我々は3種のピロリ菌 (標準株 NCTC 11637、ヒト胃がん由来株 YS 50、ヒト十二指腸潰瘍由来株 YS 27) を用いて、沢わさびの根茎と葉、茎について、それらの各エーテル抽出物がピロリ菌に対して抗菌活性を示すか否かを検定した[16,17]。いずれの抽出物もピロリ菌に対し、強い抗菌活性を示し、特に AITC 含有量の少ない葉抽出物が根茎抽出物と同程度の抗菌活性を示したことは興味深く、ITC 以外の抗菌成分が存在することが予想された (Table 2)。

空腹時には pH が1～2になる強酸性の胃内で、ピロリ菌はウレアーゼ (尿素分解酵素) により尿素を分解してアンモニアを生成し、近傍を中和して生存している。したがって、ピロリ菌の持つウレアーゼ活性を阻害すれば、ピロリ菌は胃内で生存することが困難と考えられた。我々がわさび抽出物のウレアーゼ阻害活性を検定したところ、根茎、葉、茎いずれも強い阻害活性を示した。また、含有成分である AITC が強い阻害効果を示したが、AITC 以外のウレアーゼ阻害物質の存在も示唆された。

我々はピロリ菌 (ATCC 43504) に感染したスナネズミ (MGS/Sea) を用いて、in vivo における沢わさび葉の抗ピロリ菌活性を検定した[18]。エーテル抽出物を14日間強制的に胃内に投与すると、ピロリ菌の生菌数が減少傾向を示し、胃粘膜の出血と胃粘膜障害がいずれも改善された。このことはピロリ菌培養液中に発生する $\cdot O_2^-$ をわさび葉エーテル抽出物が消去すること、in vivo 試験においても葉エーテル抽出物がピロリ菌による酸化的傷害を抑制したものと考えている。さらに、わさび葉の凍結乾燥物を固形飼料に混合して自由摂取させたスナネズミの胃粘膜では、生菌数の減少、出血の改善とともに有意な胃粘膜障害の抑制を確認した (Fig. 3)。これらの結果から、沢わさびを摂取することにより、ピロリ菌に対する感染予防や胃粘膜障害の緩和が期待できると思われる。

現在、ピロリ菌の除菌治療はプロトンポンプ阻害剤のランソプラゾールと抗生物質であるアモキシシリン、クラリスロマイシンの組み合わせが用いられており、3剤併用療法と呼ばれている。近年、これら薬剤を用いた除菌により消化器性疾患の予防措置がとられている。しか