The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Azoxystrobin at sub-cytotoxic concentrations disrupts intracellular zinc homeostasis: A flow cytometric analysis with rat thymic lymphocytes and fluorescent probes

Mai Shoji[1], Masaki Asada[1], Akihiko Matsumoto[1], Haruki Nishino[1], Ao Yi Xiang[1], Mizuki Mizobuchi[2], Naoki Kanematsu[2], Hajime Miura[1] and Norio Kamemura[2]

[1]Graduate School of Integrated Arts and Sciences, Tokushima University, Tokushima 770-8502, Japan
[2]Department of Food-Nutritional Sciences, Faculty of Life Sciences, Tokushima Bunri University, Tokushima 770-8514, Japan

(Received December 13, 2019; Accepted December 20, 2019)

ABSTRACT — Azoxystrobin is a broad-spectrum fungicide having a wide usage. However, the toxic effect of azoxystrobin in humans is not reported. In Japan, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit (2.5 mg/kg) in a shipment of an Australian barley used in different food products. Thus, there is a chance of azoxystrobin exposure through food to humans, and hence it is imperative to study the toxic effects of this compound. In this study, the toxic effect of azoxystrobin was evaluated to predict its adverse effects on human. Azoxystrobin at 3-30 μM (approximately 1.2-12.1 mg/L) raised the intracellular Zn^{2+} concentration of rat thymic lymphocytes. This increase was due to an influx of extracellular Zn^{2+} and a release of intracellular Zn^{2+}. Azoxystrobin partially inhibited the temperature-dependent Zn^{2+} influx, thus jeopardizing the cellular Zn^{2+} homeostasis. Because Zn^{2+} is an important intracellular messenger in lymphocytes, this altered Zn^{2+} homeostasis might lead to adverse effects if the blood concentration of azoxystrobin reaches 3 μM or more in humans. However, by extrapolating the azoxystrobin pharmacokinetics data of rats to human, it can be predicted that such high blood concentration may be unlikely in humans.

Key words: Azoxystrobin, Intracellular zinc, Lymphocytes, Membrane potential

INTRODUCTION

Azoxystrobin is a broad-spectrum fungicide and its overall usage is increasing every year after the start of its sale (U.S. Geological Survey, 2016). This chemical agent blocks the electron transport chain in mitochondria resulting in inhibition of ATP production and higher oxidative stress in fungus (Sauter, 1995; Wong and Wilcox, 2001). Azoxystrobin is also toxic to different vertebrates and invertebrate species of both freshwater and marine systems (Rodrigues *et al*., 2013). Therefore, toxicological studies with azoxystrobin in non-target organisms involving mammals and particularly human draw attention (European Food Safety Authority, 2010; Kunz *et al*., 2017). To the best of our knowledge, there is no existing data to demonstrate the toxic effects of azoxystrobin at environmental concentrations in human. However, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit (2.5 mg/kg) in a shipment of an Australian barley (Minister of Agriculture, Forestry, and Fisheries of Japan, 2018). This particular barley was used in different food products and the manufacturing company voluntarily recalled its cereal products. In this study, the toxic effect of azoxystrobin was evaluated in an

Correspondence: Norio Kamemura (E-mail: kamemura.norio@tks.bunri-u.ac.jp)

in vitro model to predict its adverse effects on human.

Increased oxidative stress is found to be one of the adverse effects of azoxystrobin in some species of vertebrates and invertebrates (Rodrigues *et al*., 2013; Han *et al*., 2014, 2016). Oxidative stress can mobilize intracellular zinc (Zn^{2+}) (McCord and Aizenman, 2014; Abiria *et al*., 2017). Therefore, there is a possibility that azoxystrobin leads to intracellular Zn^{2+} mobilization by oxidative stress resulting in the elevation of intracellular Zn^{2+} concentration. Zn^{2+} is released during the oxidative conversion of intracellular non-protein thiol to disulfide (Maret, 1995). To test this possibility, the effect of azoxystrobin on cellular content of non-protein thiols [(NPT)i] and intracellular Zn^{2+} concentration [(Zn^{2+})i] were studied using a flow cytometric technique coupled with appropriate fluorescent probes in lymphocytes isolated from rat thymus (thymocytes). Along with azoxystrobin, another postharvest fungicide pyrimethanil was also studied at the same concentration. It is to be mentioned that Zn^{2+} enters rat thymic lymphocytes through a temperature-sensitive pathway. The application of $ZnCl_2$ significantly increases the (Zn^{2+})i and this phenomenon is completely suppressed in cold condition (Takahashi *et al*., 2012; Fukunaga *et al*., 2015). The thymus gland is very active in the pre-adolescent and neonatal periods. Since there is a concern regarding the adverse effect of azoxystrobin in the pediatric population, any information pertaining to the adverse cellular changes in thymocytes by azoxystrobin may be of clinical importance.

MATERIALS AND METHODS

Chemicals

Azoxystrobin (standard purity of 99.8%) and pyrimethanil (standard purity of 98.9%) were procured from Wako Pure Chemicals (Tokyo, Japan). Propidium iodide (PI), 5-chloromethylfluorescein diacetate (5-CMF-DA), and FluoZin-3-AM were obtained from Invitrogen (Eugene, OR, USA). Diethylenetriamine-N,N,N',N'',N''-pentaacetic acid (DTPA; Dojindo Molecular Technologies, Inc.) and *N*,*N*,*N'*,*N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; Dojindo Molecular Technologies, Inc.) were used as extracellular and intracellular Zn^{2+} chelators respectively. The other reagents were obtained from Wako Pure Chemicals (Tokyo, Japan).

Cell preparation

The animal experiments were performed after obtaining approval from the committee of Tokushima University, Tokushima, Japan (approval no. 05279). The thymus glands of male Wistar rats (6-8-week-old) were triturated in chilled Tyrode's solution (2-4°C) to dissociate thymic lymphocytes (thymocytes). The solution containing thymocytes (cell suspension) was stored at 36-37°C for at least 1 h before using in the subsequent experiments. It was noted that the cell suspension contained a small amount (216.9 ± 14.4 nM) of Zn^{2+} from the cell preparation (Sakanashi *et al*., 2009).

Thymocytes were used in this experiment for chemical cytotoxicity because these living cells with intact cell membranes can be easily isolated from thymus without enzymatic treatment. However, there is a technical limitation to obtain freshly dissociated thymocytes. During prolonged incubation, some cells may spontaneously undergo apoptosis. Hence, all the experimental works were completed within 6-7 hr after organ dissection to avoid spontaneous apoptosis of the cells. Since cell shrinkage is one of the parameters detected in the early stage of apoptosis (Bortner and Cidlowski, 1998), the forward scatter was used as a parameter to determine the cell size. The forward scatter and cell viability in control cells were kept unchanged during the entire experiment. Azoxystrobin (0.3-100 mM in 2 µL dimethyl sulfoxide) was added to the suspension (1998 µL) to prepare bath concentrations of 0.3-100 µM and the cells were treated with azoxystrobin for 1 hr. To test the effect of azoxystrobin on the temperature-sensitive Zn^{2+} pathway, the augmentation of FluoZin-3 fluorescence by applying 3 µM $ZnCl_2$ was examined in the presence and absence of 30 µM azoxystrobin.

Dimethyl sulfoxide (up to 0.3%) did not affect cell viability and the fluorescent cellular parameters studied. A sample (100 µL) from each cell suspension was analyzed by flow cytometry. Data acquisition from 2500-3000 cells took approximately 10-15 sec. The cellular parameters monitored for 2500 cells were similar to those examined for 10000 cells. Thus, 2500-3000 cells were deemed sufficient to study the cellular actions of azoxystrobin.

Measurements of cellular parameters with fluorescent probes

Zn^{2+} at a micromolar concentration significantly potentiates the cytotoxic action of H_2O_2 (Matsui *et al*., 2010). To examine whether azoxystrobin also has a similar property, the increase in the percentage of cell population exhibiting PI fluorescence by 300 µM H_2O_2 was compared in the presence and absence of 30 µM azoxystrobin.

Fluorescence was analyzed using a flow cytometer and software package (CytoACE-150; JASCO, Tokyo, Japan). Azoxystrobin at concentrations used in this study exhibited no fluorescence under the said experimental condi-

tions. PI (5 µM) was added to the suspension to assess cell lethality (percentage population of cells exhibiting PI fluorescence). The measurement of cellular non-protein thiols (NPT)i was performed using 1 µM 5-CMF-DA (Chikahisa *et al*., 1996). In order to monitor the change in intracellular Zn^{2+} concentration (Zn^{2+})i, the cells was incubated with 1 µM FluoZin-3-AM (Gee *et al*., 1989) for 50-60 min before initiating estimation.

FluoZin-3 fluorescence was monitored in the cells under extracellular Ca^{2+}-free condition because azoxystrobin also increases Ca^{2+} influx, as observed in our previous experiments (unpublished data on file). Azoxystrobin-induced changes in FluoZin-3 and 5-CMF fluorescence were examined only in the living cells with intact cell membranes that did not exhibit PI fluorescence. The excitation wavelength of all the probes (PI, 5-CMF, and FluoZin-3) was 488 nm, and emissions were detected at 530 ± 20 nm for 5-CMF and FluoZin-3, and at 600 ± 20 nm for PI. Each experimental series was conducted thrice unless stated otherwise.

Statistical analysis

The continuous variables were expressed as mean and standard deviation. Data were analyzed using Tukey's multivariate method and a p value of < 0.05 was considered as statistically significant

RESULTS

Impact on cell lethality by azoxystrobin and pyrimethanil

Treatment with 30 µM azoxystrobin and 30 µM pyrimethanil for 3 hr did not increase the population of cells exhibiting PI fluorescence, dead cells or cells with compromised cell membranes (Fig. 1). Thus, both these agents at 30 µM can be assumed to be non-cytotoxic.

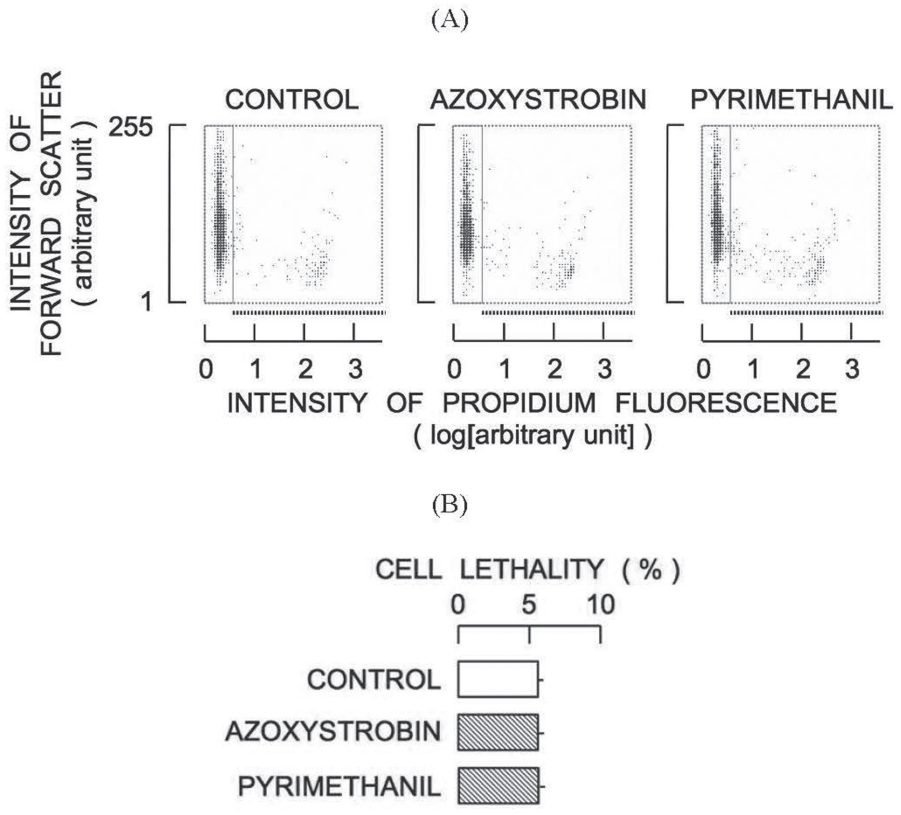

Fig. 1. Cytotoxic action of azoxystrobin on rat thymocytes. A: Changes in cytogram (forward scatter versus intensity of propidium fluorescence) after incubation with 30 µM azoxystrobin and 30 µM pyrimethanil for 3 hr. The cytogram consisted of 2000 cells. The dotted line under the cytogram indicates the cells exhibiting propidium fluorescence. B: The percentage population of cells exhibiting propidium fluorescence after 3 hr incubation with 30 µM azoxystrobin and 30 µM pyrimethanil. The column and bar indicate the mean and standard deviation of four samples.

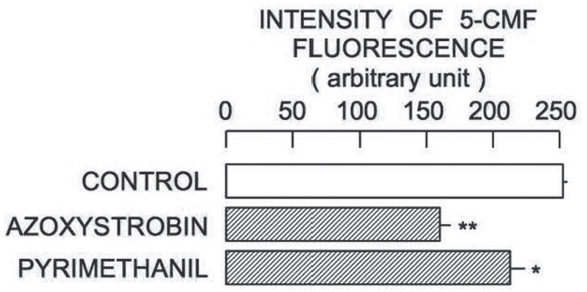

Fig. 2. Azoxystrobin-induced attenuation of 5-CMF fluorescence in rat thymocytes. The effects were examined at 2 hr after application of 30 μM azoxystrobin and 30 μM pyrimethanil. The column and bar indicate the mean intensity of 5-CMF fluorescence and its standard deviation of four samples. *$p < 0.05$, **$p < 0.01$ when compared with the control.

Changes in 5-CMF fluorescence by azoxystrobin and pyrimethanil

Although the cells were considered to be intact, there was a possibility that azoxystrobin and pyrimethanil at 30 μM concentration might affect some cellular parameters. To test this possibility, the effects of azoxystrobin and pyrimethanil on the (NPT)i were examined using 5-CMF-DA. 5-CMF fluorescence was significantly attenuated by the treatment with 30 μM azoxystrobin and 30 μM pyrimethanil for 3 hr (Fig. 2). The potency of azoxystrobin to reduce the (NPT)i was found to be greater than that of pyrimethanil.

Changes in FluoZin-3 fluorescence by azoxystrobin and pyrimethanil

The decrease in (NPT)i by azoxystrobin and pyrimethanil might indicate the conversion of thiols to disulfide leading to intracellular Zn^{2+} release from the cellular zinc

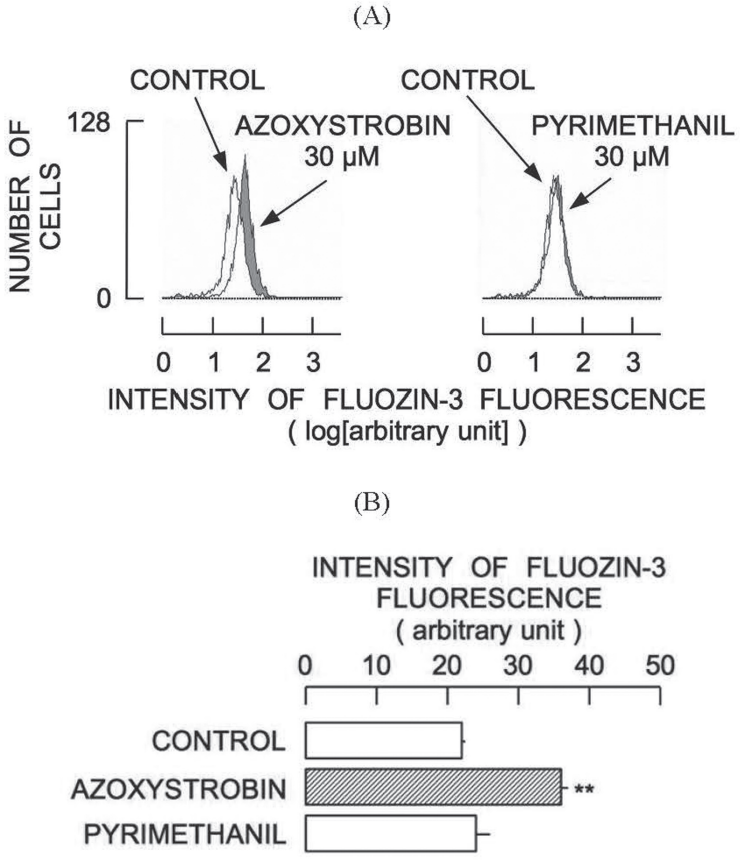

Fig. 3. Azoxystrobin-induced changes in FluoZin-3 fluorescence of rat thymocytes. A: Shift of FluoZin-3 fluorescence histogram due to application of 30 μM azoxystrobin and 30 μM pyrimethanil. The histogram was prepared with 2500 cells. The effect was examined 1 hr after the drug application. B: Changes in the mean intensity of FluoZin-3 fluorescence by 30 μM azoxystrobin and 30 μM pyrimethanil. The column and bar indicate the mean and standard deviation of four samples. ** **$p < 0.01$ when compared with the control.

stores. Therefore, the change of (Zn^{2+})I by azoxystrobin and pyrimethanil was estimated with FluoZin-3 fluorescence. The intensity of FluoZin-3 fluorescence was significantly increased by treatment with 30 μM azoxystrobin for 1 hr, but not by 30 μM pyrimethanil (Fig. 3). The FluoZin-3 fluorescence was significantly augmented by incubation with 3-30 μM azoxystrobin for 1-2 hr in a concentration-dependent manner (Fig. 4). The concentration of FluoZin-3 fluorescence observed at 1 hr after initiating incubation with azoxystrobin was similar to that observed at 2 hr.

Characteristics of azoxystrobin-induced augmentation of FluoZin-3 fluorescence

It was speculated that (Zn^{2+})i increased in the presence of 3-30 μM azoxystrobin. Therefore, the supply of increased (Zn^{2+})i was prevented by the use of DTPA, a chelator for extracellular Zn^{2+}. The concentration of FluoZin-3 fluorescence in the control was significantly reduced by 10 μM DTPA. The addition of 30 μM azoxystrobin, however, still augmented the FluoZin-3 fluorescence under extracellular Zn^{2+}-free conditions (Fig. 5). The intracellular Zn^{2+} chelator TPEN at 10 μM completely suppressed the FluoZin-3 response, which was elicited by azoxystrobin. Therefore, it is likely that the azoxystrobin-induced increase in (Zn^{2+})i was due to an influx of extracellular Zn^{2+} as well as a release of intracellular Zn^{2+}. The augmentation of FluoZin-3 fluorescence by $ZnCl_2$ in the presence of azoxystrobin was significantly less than that in the absence of azoxystrobin (Fig. 6). Thus, azoxystrobin seems to possess an inhibitory action on temperature-sensitive Zn^{2+} influx.

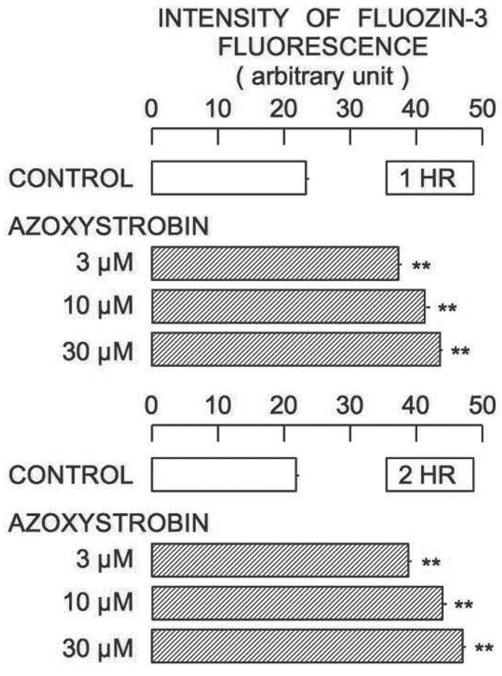

Fig. 4. Time- and concentration-dependent changes in the intensity of FluoZin-3 fluorescence by 3-30 μM azoxystrobin under extracellular Ca^{2+}-free conditions. The effects of azoxystrobin were examined at 1 hr (upper panel: 1 HR) and 2 hr (lower panel: 2 HR) after the drug application. The column and bar indicate the mean and standard deviation of four samples. $^{**}p < 0.01$ when compared with the corresponding control.

Increase in cell vulnerability to oxidative stress by azoxystrobin

The increase in the percentage of cell population exhibiting PI fluorescence by 300 μM H_2O_2 was compared in the presence and absence of 30 μM azoxystrobin. The simultaneous application of azoxystrobin and H_2O_2 increased the population of PI-stained cells than azoxystrobin alone (Fig. 7).

DISCUSSION

Azoxystrobin-induced increase in intracellular Zn^{2+} concentration

The elevation of (Zn^{2+})i may be a common characteristic of toxin-induced oxidative stress. The intracellular Zn^{2+} release is linked with Zn^{2+}-thiol/disulfide interchange (Maret, 1994) and oxidative stress converts thiols to disulfides resulting in a Zn^{2+} release. Azoxystrobin has been shown to induce oxidative stress in some vertebrates and invertebrate species (Rodrigues *et al*., 2013; Han *et al*., 2014, 2016). Therefore, it is not surprising that azoxystrobin increased (Zn^{2+})i even under extracellular Zn^{2+}-free conditions. However, it may be interesting to note here that a temperature-sensitive Zn^{2+} influx that resulted by adding $ZnCl_2$ to the cell suspension was partially suppressed by azoxystrobin. The Zn^{2+} transporters of cell and organelle membranes regulate intracellular Zn^{2+} homeostasis (Hara *et al*., 2011), and azoxystrobin may inhibit one of them. Zn^{2+} is an intracellular signaling molecule in the lymphocytes (Hirano *et al*., 2008; Fukuda *et al*., 2011), and therefore, azoxystrobin is considered to jeopardize some cellular functions of lymphocytes resulting in immune malfunctions.

Implication of this work

The use of azoxystrobin is increasing every year (U.S. Geological Survey, 2016). In Japan, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit of (2.5 mg/kg) in a shipment of an Aus-

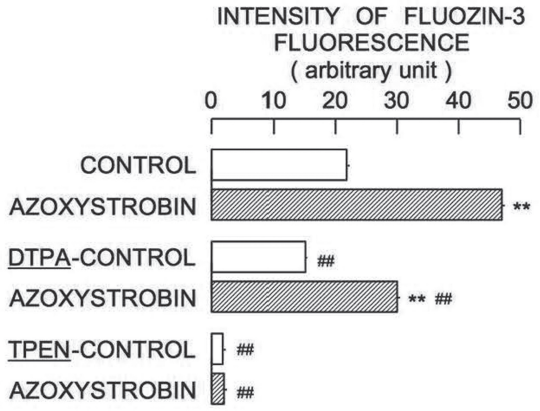

Fig. 5. Change in intensity of FluoZin-3 fluorescence by azoxystrobin in the presence of Zn^{2+} chelator (DTPA and TPEN). Paired columns (empty: control and filled: azoxystrobin) indicate the group of cells treated without and with 30 μM azoxystrobin in the presence or absence of respective Zn^{2+} chelator (DTPA for extracellular Zn^{2+} and TPEN for intracellular Zn^{2+}). $**p < 0.01$ between the group of cells treated with azoxystrobin and control. $\#\#p < 0.01$ between control without Zn^{2+} chelator (top control) and other test groups.

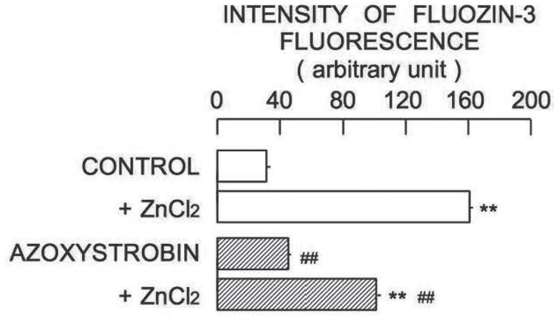

Fig. 6. Effect of azoxystrobin on augmentation of FluoZin-3 fluorescence by the addition of 3 μM $ZnCl_2$ (upper pair: change of FluoZin-3 fluorescence by $ZnCl_2$, lower pair: change by $ZnCl_2$ in the presence of 30 μM azoxystrobin). The column and bar indicate the mean and standard deviation of four samples. $**p < 0.01$ between the group of cells treated with $ZnCl_2$ and control. $\#\#p < 0.01$ between control without azoxystrobin and groups with azoxystrobin.

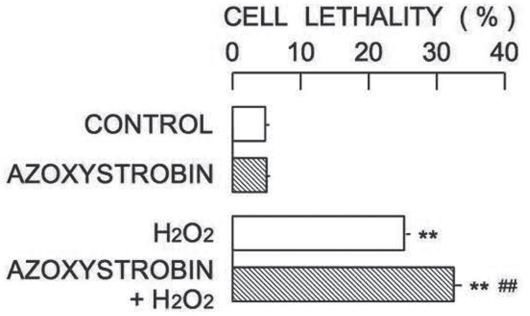

Fig. 7. Changes in cell lethality in the absence (control) and presence of 30 μM azoxystrobin, 300 μM H_2O_2, and their combination (azoxystrobin and H_2O_2). The effects were examined at 4 hr after the drug application. The column and bar indicate the mean and standard deviation of four samples. $**p < 0.01$ when compared with the control. $\#\#p < 0.01$ between cells treated with H_2O_2 in the presence and absence of azoxystrobin.

tralian barley used in different food products (Minister of Agriculture, Forestry, and Fisheries of Japan, 2018). In this *in vitro* study, the lowest concentration of azoxystrobin to increase $(Zn^{2+})i$ was found to be 3 μM (equivalent to 1.2 mg/L). As azoxystrobin pharmacokinetic data in human and azoxystrobin amount in food products using barley are not available, it may not be appropriate to consider that the blood concentration of azoxystrobin will reach to 1.2 mg/L in human consumers. The pharmacokinetics of oral azoxystrobin was studied in rats (Shah and Ray, 2008), and it was revealed that more than 85% of the administered azoxystrobin gets excreted unchanged within 48 hr in feces (up to 80%) and urine (< 17%). Therefore, the oral absorption of azoxystrobin is low, and it also undergoes extensive metabolism. After extrapolating these data to human, it can be predicted that the blood concentration of azoxystrobin may not get too high after intake of foods containing azoxystrobin. Hence, an adverse effect from azoxystrobin in human is unlikely.

Conflict of interest---- The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

The Japan Society for the Promotion of Science (Tokyo, Japan) provided a Grant-in-Aid for Scientific Research (C26340039) that was used in this study. We thank Yasuo Oyama for skillful assistance.

REFERENCES

Abiria, S.A., Krapivinsky, G., Sah, R., Santa-Cruz, A.G., Chaudhuri, D., Zhang, J., Adstamongkonkul, P., DeCaen, P.G. and Clapham, D.E. (2017): TRPM7 senses oxidative stress to release Zn^{2+} from unique intracellular vesicles. Proc. Natl. Acad. Sci. USA, **114**, E6079-E6088.

Chikahisa, L., Oyama, Y., Okazaki, E. and Noda, K. (1996): Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Jpn. J. Pharmacol., **71**, 299-305.

Bortner, C.D. and Cidlowski, J.A. (1998): A necessary role for cell shrinkage in apoptosis. Biochem. Pharmacol., **56**, 1549-1559.

European Food Safety Authority. (2010): Conclusion on the peer review of the pesticide risk assessment of the active substance azoxystrobin. EFSA J., **8**, 1542.

Fukada, T., Yamasaki, S., Nishida, K., Murakami, M. and Hirano, T. (2011): Zinc homeostasis and signaling in health and diseases. J. Biol. Inorg. Chem., **16**, 1123-1134.

Fukunaga, E., Oyama, T.M. and Oyama, Y. (2015): Elevation of the intracellular Zn^{2+} level by 2-n-octyl-4-isothiazolin-3-one in rat thymocytes: an involvement of a temperature-sensitive Zn^{2+} pathway. Toxicol. Res., **4**, 65-70.

Gee, K.R., Zhou, Z.L., Qian, W.J. and Kennedy, R. (2002): Detection and imaging of zinc secretion from pancreatic β-cells using a new fluorescent zinc indicator. J. Am. Chem. Soc., **124**, 776-778.

Han, Y., Liu, T., Wang, J., Wang, J., Zhang, C. and Zhu, L. (2016): Genotoxicity and oxidative stress induced by the fungicide azoxystrobin in zebrafish (Danio rerio) livers. Pestic. Biochem. Physiol., **133**, 13-19.

Han, Y., Zhu, L., Wang, J., Wang, J., Xie, H. and Zhang, S. (2014): Integrated assessment of oxidative stress and DNA damage in earthworms (Eisenia fetida) exposed to azoxystrobin. Ecotoxicol. Environ. Saf., **107**, 214-219.

Hara, T., Takeda, T.A., Takagishi, T., Fukue, K., Kambe, T. and Fukada, T. (2017): Physiological roles of zinc transporters: molecular and genetic importance in zinc homeostasis. J. Physiol. Sci., **67**, 283-301.

Hirano, T., Murakami, M., Fukada, T., Nishida, K., Yamasaki, S. and Suzuki, T. (2008): Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Adv. Immunol., **97**, 149-176.

Kunz, J.L., Ingersoll, C.G., Smalling, K.L., Elskus, A.A. and Kuivila, K.M. (2017): Chronic toxicity of azoxystrobin to freshwater amphipods, midges, cladocerans, and mussels in water only exposures. Environ. Toxicol. Chem., **36**, 2308-2315.

Maret, W. (1995): Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. Neurochem. Int., **27**, 111-117.

Matsui, H., Oyama, T.M., Okano, Y., Hashimoto, E., Kawanai, T. and Oyama, Y. (2010): Low micromolar zinc exerts cytotoxic action under H_2O_2-induced oxidative stress: excessive increase in intracellular Zn^{2+} concentration. Toxicol., **276**, 27-32.

McCord, M.C. and Aizenman, E. (2014): The role of intracellular zinc release in aging, oxidative stress, and Alzheimer's disease. Front. Aging Neurosci., **6**, 77.

Minister of Agriculture, Forestry, and Fisheries of Japan, 2018. Press Release; On the Food Sanitation Act violation concerning imported barley from Australia (in Japanese) http://www.maff.go.jp/j/press/seisaku_tokatu/boeki/180403.html

Rodrigues, E.T., Lopes, I. and Pardal, M.Â. (2013): Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environ. Int., **53**, 18-28.

Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y. and Oyama, Y. (2009): Zn^{2+} derived from cell preparation, partly attenuates Ca^{2+}-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. Toxicol. In Vitro, **23**, 338-345.

Sauter, H., Ammerman, E., Benoit, R., Brand, S., Gold, R.E., Grammenos, W., Köhl, H., Lorenz, G., Muller, B., Röhl, F., Schirmer, U., Speakman, J.B., Wenderoth, B. and Wingert, H. 1995. Mitochondrial respiration as a target for antifungals: Lessons learned from research on strobilurins. In Antifungal Agents—Discovery and Mode of Action; Dixon, G.K., Copping, L.G., Hollowman, D.W., Eds. BIOS: Oxford, UK. 173-191.

Shah, P.V. and Ray, D. 2008. Azoxystrobin. In Pesticide residues in food-2008, Joint Meeting of Food and Agriculture Organization of the United Nations and World Health Organization, pp. 3-34. http://www.inchem.org/documents/jmpr/jmpmono/v2008pr01.pdf

Takahashi, Y., Kanemaru, K., Imai, S., Miyoshi, N., Kawanai, T. and Oyama, Y. (2012): Yttrium decreases the intracellular Zn^{2+} concentration in rat thymocytes by attenuating a temperature-sensitive Zn^{2+} influx. Environ. Toxicol. Pharmacol., **34**, 574-578.

U.S. Geological Survey. Pesticide National Synthesis Project, 2016. Azoxystrobin https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2015&map=AZOXYSTROBIN&hilo=L&disp=Azoxystrobin

Wong, F.P. and Wilcox, W.F. (2001): Comparative physical modes of action of azoxystrobin, mancozeb, and metalaxyl against Plasmopara viticola (grapevine downy mildew). Plant Dis., **85**, 649-656.