FULL PAPER *Pathology*

Comparison of Enterotoxicity between Autumn Crocus (*Colchicum autumnale* L.) and Colchicine in the Guinea Pig and Mouse : Enterotoxicity in the Guinea Pig Differs from That in the Mouse

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ABSTRACT. Autumn crocus poisoning of cattle is characterized by severe diarrhea caused by alkaloid colchicine. Previously, we examined pathologically this poisoning in cattle and reported that enterotoxic lesions were closely associated with apoptosis. To examine enterotoxicity of autumn crocus more precisely, a reproductive study was performed using guinea pigs and mice, and pathological findings associated with autumn crocus poisoning were compared with those of colchicine. Each group of guinea pigs given the bulb of autumn crocus or colchicine exhibited severe diarrhea. Histopathological findings in intoxicated guinea pigs were entirely consistent with those in the autumn crocus-poisoned cattle. In contrast, each group of mice administered with the bulb or colchicine did not develop diarrhea. Our results confirmed that the toxicity of autumn crocus bulb is attributable to the toxicity of ingredient colchicine, and revealed that the guinea pig has high reproducibility of autumn crocus poisoning in cattle and colchicine poisoning in humans. It has been reported that the physiological mechanism of the apoptotic process for eliminating the enterocytes in the mouse and rat differs from that of the guinea pig, monkey, cattle and horse. Taking the observation that the former animals do not develop diarrhea, whereas the latter animals do so in the autumn crocus or colchicine poisoning into consideration, it would seem that the species-difference in enterotoxicity of autumn crocus may be closely associated with the physiological mechanism of eliminating the effete enterocytes.

KEY WORDS: apoptosis, autumn crocus (*Colchicum autumnale* L.), colchicine, guinea pig, mouse.

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Autumn crocus (*Colchicum autumnale* L.), which is a perennial plant classified as a member of the *Liliaceae* family, has been reported to be toxic to many animal species [3–5, 15, 19]. It has been reported that acute poisoning by autumn crocus is characterized by acute lethal diarrhea in cattle, sheep and horses[4, 15]. In previous papers, we reported that tissue destruction was characterized by the presence of numerous apoptotic cells and mitotically arrested cells in the alimentary epithelial layer of cattle experimentally administered with autumn crocus bulb [18, 19]. It has been reported that autumn crocus poisoning is caused by the ingredient alkaloid colchicine [4] and our previous studies supported the hypothesis [18, 19]. Thus far, however, no comparison has been made between enterotoxicities of autumn crocus and colchicine. Therefore, the actual cause of autumn crocus poisoning remains to be determined.

A trial to reproduce intestinal disorder of autumn crocus poisoning of cattle in mice as a model animal was unsuccessful in inducing diarrhea in intoxicated mice (unpublished data). A species-difference has also been reported in toxicity of colchicine [16]. Although colchicine has long been used to alleviate the painful symptoms of human gout, severe diarrhea almost invariably occurred [2, 16]. A failure in inducing diarrhea has also been reported on rat dosed on colchicine [16].

We reported in a previous paper that intestinal tissue destruction caused by autumn crocus was closely associated with apoptosis [19]. It has been reported that the mechanisms of the apoptotic process to eliminate the effete enterocytes in the mouse and rat differed from those in the guinea pig, monkey, cattle and horse [8, 9, 11–13]. We paid attention to the prior observations that the former animals do not develop diarrhea, while the latter animals develop diarrhea in the autumn crocus or colchicine poisoning [2, 4, 15, 16, 18]. We therefore assumed that autumn crocus poisoning of guinea pig might result in lethal diarrhea, presenting similarity to autumn crocus poisoning of cattle.

Based on such a background, we undertook the present study using guinea pigs and mice as the experimental animals to demonstrate species-difference in enterotoxicity of autumn crocus and colchicine.

MATERIALS AND METHODS

Agents: The alkaloid colchicine levels contained in the commercially obtained crude autumn crocus bulbs were chemically determined by high performance liquid chromatography (HPLC) [20], as a result, the crocus bulb used in this study contained 0.04% colchicine. The pounded bulb was made up in 0.9% sterile saline immediately before use. Colchicine ($C_{22}H_{25}NO_6$ Sigma Chemical Co. U.S.A.) was made up in 0.9% sterile saline immediately before use.

Experimental design: Male Hartley guinea pigs aged 3 weeks and male Std:ddy mice aged 6 weeks were purchased from Japan SLC Co. (Shizuoka, Japan). Eight guinea pigs and eight mice were orally administered with 10 g/kg of pounded autumn crocus bulb by gastric intubation. Five guinea pigs and five mice were euthanized 6 hr after administration and the remaining three guinea pigs and three mice were euthanized 12 hr after administration. Five guinea pigs and five mice were orally given colchicine 4 mg/kg to attain the same

level as colchicine contained in the crocus bulb and they were euthanized 6 hr after administration. Three guinea pigs and three mice which were given vehicle alone were used as controls. After administration, all animals were carefully observed. For electron microscopy, the animals euthanized 6 hr after administration were utilized.

All animals were anesthetized with diethyl ether, perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and immediately necropsied. Portions of the intestinal tissue, the duodenum (a region of 10 mm distal to the pylorus), the jejunum (a region of 10 mm around the middle portion of the small intestine), the ileum (a region of 10 mm proximal to the cecum), the colon (a region of 10 mm distal to the cecum), and the rectum (a region of 10 mm proximal to the anus), were collected.

Histological examination: The tissue samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, embedded in paraffin and sectioned at 4 μm. Dewaxed sections were stained with hematoxylin and eosin (HE).

To detect cells with DNA strand break in the apoptotic process [14, 17], paraffin sections of the intestine were examined by the terminal deoxynucleotidyl transferase (TdT) -mediated dUTP-nick end labeling (TUNEL) procedure [7]. After treatment with proteinase K for 20 min at 37°C and then with 3% hydrogen peroxide for 20 min at room temperature to quench endogenous peroxidase activity, the sections were incubated with TdT (Takara, Japan) and biotinylated dUTP (Boehringer Mannheim Biochemica) in TdT buffer (sodium cacodylate, 100 mM; cobalt chloride, 1 mM, pH 7.0) for 90 min at 37°C. Subsequently, the sections were blocked with 2% bovine serum albumin for 10 min at room temperature, and then treated with peroxidase-labeled streptavidin for 30 min at room temperature. Peroxidase was detected with DAB. The sections were counterstained lightly with 5% methylgreen. In negative controls, distilled water replace the TdT enzyme.

Ultrastructural examination: For electron microscopy, parts of the intestinal tissue removed as mentioned above were cut into small pieces and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hr, postfixed in 1% osmium tetroxide and embedded in resin. Ultrathin sections were stained with saturated uranyl acetate and lead citrate and examined with an H-7500 electron microscope (Hitachi Co., Ltd., Tokyo) at 80 kV.

Frequency of apoptotic cells and mitotically arrested cells in the intestinal crypt: The distribution of apoptotic cells and mitotically arrested cells along the cryptal length was compared to characterize the target cell of the toxic agent. The cells in M phase, including pro-, meta-, ana-, and telophase, were counted as mitotic cells. The cells having round or ovoid cytoplasmic masses with or without nuclear material were counted as apoptotic cells. Cells with large condensed chromatin masses were counted as mitotically arrested cells. The apoptotic and mitotically arrested cells were calculated in 200 randomly selected crypts in which a continuous single row of epithelial cells could be seen from the bottom to the top of the crypt. Mean values ± standard deviation (SD) per crypt were calculated for each group. The numbers of apoptotic cells and mitotically arrested cells in the small intestine were compared with those in the large intestine by means of Student's *t*-test. Values of <0.05 were considered significant.

RESULTS

Clinical findings and gross examination: All intoxicated guinea pigs exhibited similar clinical signs of depression, eventual diarrhea and weakness. Diarrhea developed 5 hr after administration and the symptoms progressively worsened. On the other hand, all mice, both treated and control, appeared essentially normal in behavior following exposure to the bulb and colchicine. None of mice develop diarrhea during the course of the experiment. At necropsy, all guinea pigs showed similar changes characterized by hemorrhage and congestion on the serosal membrane of the intestinal tract. In the intoxicated mice, similar changes were observed, but they were slighter than those of guinea pigs.

Histopathological findings: All guinea pigs administrated with the bulb showed similar changes upon histological examination. In the small intestine, villous atrophy accompanied by villous fusion was observed at almost every part examined (Fig. 1b). Conspicuous changes in the alimentary epithelium included cellular destruction showing karyopyknosis, karyorrhexis and mitotically arrested cells (Fig. 2). In guinea pigs euthanized 12 hr after administration, the tissue destruction and villous atrophy were severe (Fig. 1c). The lesions in the colchicine-administered guinea pigs were similar to those in the bulb-fed ones.

Lesions in both colchicine-administered and bulb-fed mice were similar to those found in guinea pigs received colchicine or bulb except for the absence of villous atrophy. The tissue destructions of the colchicine-administered and bulb-fed mice euthanized 12 hr after administration were more severe than those euthanized 6 hr after administration, however, villous atrophy did not appear yet (Fig. 1 e, f).

In the small intestine of control guinea pigs, dense clusters of TUNEL-positive cells were observed only in the lamina propria of villous tip, and there were no positively stained cells in the crypts and lumia. In contrast, positive cells were numerous, involving the intestinal epithelia in both crypt and atrophied villi of bulb- or colchicine-intoxicated guinea pigs. These positively stained cells appeared to be corresponded to those cells that had karyopyknotic or karyorrhectic nuclei as observed by light and electron microscopy. Cells with picnotic nuclei exfoliated into the lumen also reacted positively, while cells undergoing arrested mitosis showed a negative reaction.

In the small intestine of the control mice, TUNEL-positive cells were scattered in the epithelial layer of villi, and there were no positively stained cells in the crypt. In mice given the bulb or colchicine, positive cells were numerous, involving the intestinal epithelia in both crypts and villi, consistenting with pyknotic cells. Mitotically arrested cells reacted negatively, as observed in the guinea pigs.

Ultrastructural findings: Among the crypt epithelial cells in guinea pigs administered with bulb or colchicine, there

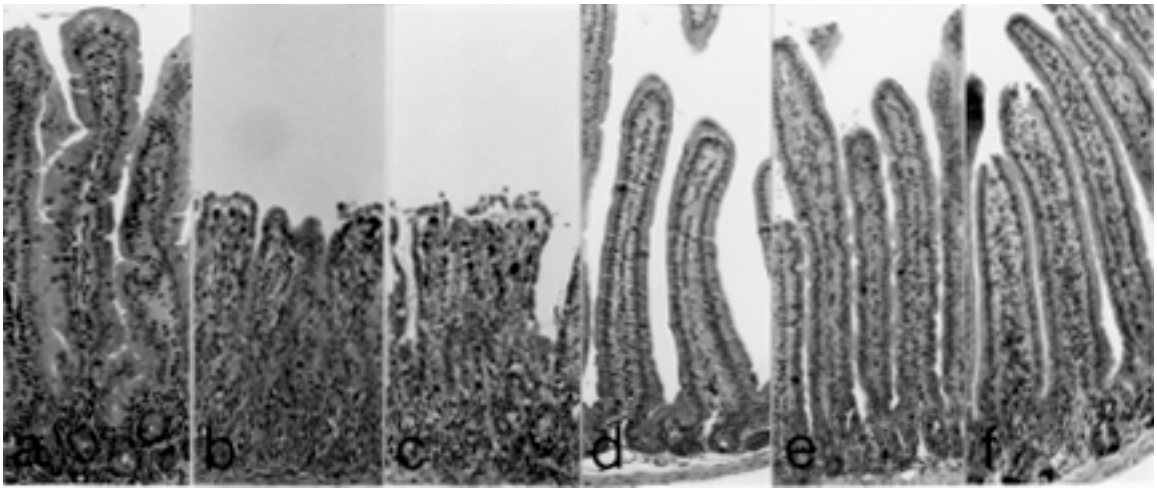

Fig. 1 (a-f). Small intestine. Comparison of histological findings in a guinea pig received autumn crocus bulb (b&c) with a control guinea pig (a) and in a mouse given autumn crocus bulb (e&f) with a control mouse (d). HE. × 95. In the guinea pig euthanized 6 hr after administration, villous atrophy with villous fusion is seen (b) in comparison with the control guinea pig (a). Note destruction of the crypt epithelium. In the guinea pig euthanized 12 hr after administration, villous atrophy and destruction of the crypt epithelium are severe (c). In both mice euthanized 6 hr (e) and 12 hr after administration (f), karyopyknosis and mitotically arrested cells are seen in the crypt, but villus remains intact.

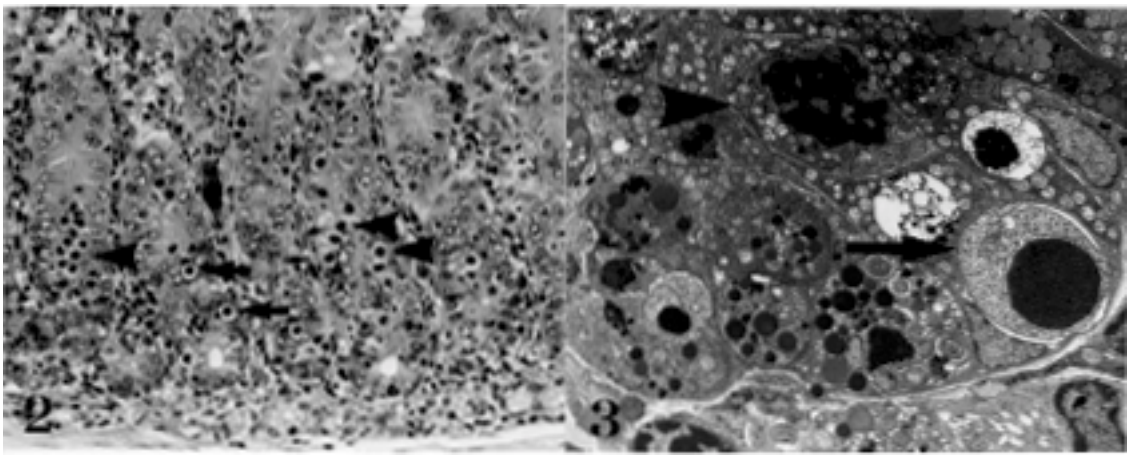

Fig. 2. Small intestine. A guinea pig administered with autumn crocus bulb. Numerous apoptotic cells (arrows) and mitotically arrested cells (arrowheads) are observed in the crypt epithelial cells. HE. × 200.

Fig. 3. Transmission electron micrograph of the small intestine of a guinea pig administered with autumn crocus bulb. Apoptotic cell (arrow) and mitotically arrested cell (arrow head) in the intestinal crypt. × 2,700.

were numerous apoptotic cells with condensed chromatin mass and atrophied cytoplasm (Fig. 3). Mitotically arrested cells had the swollen cytoplasm, condensed nuclear chromatine mass, and dilated endoplasmic reticulum, and they were located in the intestinal crypt epithelial layer. Control guinea pigs had neither apoptotic cells nor mitotically arrested cells in the crypt.

In mice administered with bulb or colchicine, apoptotic cells and mitotically arrested cells were seen in the crypt epithelium in the same way as seen in the bulb- or colchicine-intoxicated guinea pigs.

Frequency and distribution of apoptotic cells and mitotically arrested cells in the crypt: The frequency of mitotic cells per crypt in the control guinea pig and control mouse is illustrated in Fig. 4a. The number of normal mitotic cells in the small intestine was greater than that in the large intestine in both animals ($p<0.05$).

The frequencies of apoptotic cells and mitotically arrested cells per crypt following administration of the bulb or colchicine are illustrated in Figs. 4b-e. Apoptotic cells appeared with greater frequency in the small intestine than in the large intestine ($p<0.05$).

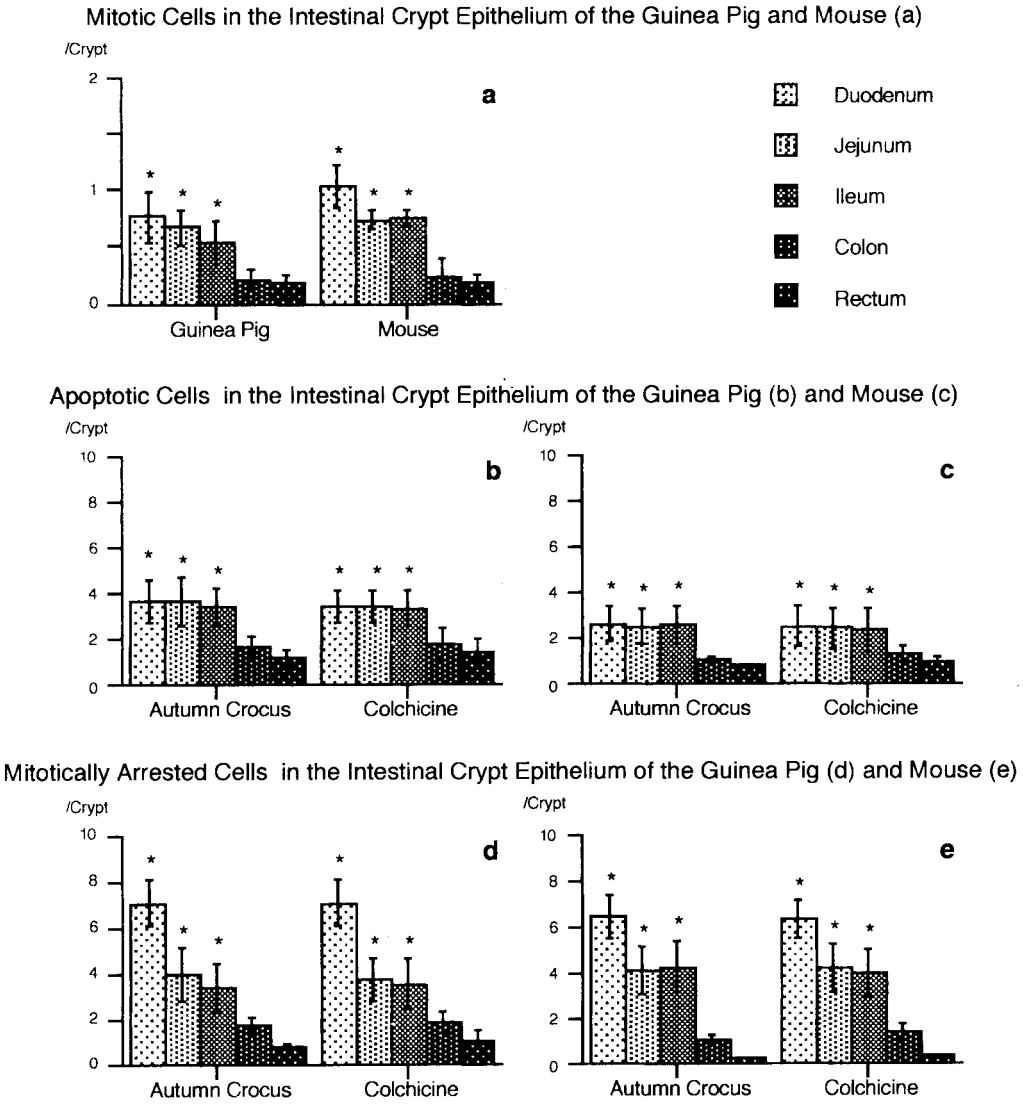

Fig. 4 (a-e). Frequency of mitotic cells per crypt in the control animals (a) and that of apoptotic cells (b, c) and mitotically arrested cells (d, e) per crypt following administration of the bulb and colchicine. Guinea pig (b, d). Mouse (c, e). Error bar = S.D. *; Significantly different from the large intestine ($p<0.05$).

DISCUSSION

In the present investigation, guinea pigs and mice were administered with autumn crocus bulb or colchicine at the almost equivalent toxic level, and toxic apoptotic changes in the intestines were comparatively studied in the animals euthanized 6 hr and 12 hr after administration. The results presented here indicated that the sensitivity to both bulb and colchicine was higher in guinea pigs than in mice, while intoxicated signs and lesions in both animals were almost identical. There were no noticeable differences between the bulb-fed and colchicine-administered groups, substantiating the hypothesis that autumn crocus poisoning is caused by ingredient colchicine. Species-difference in response to the toxin was manifested in diarrhea and villous atrophy in the small intestine in guinea pigs. In contrast, mice received the bulb or colchicine did not develop diarrhea. Histologically, despite the presence of characteristic cellular destruction consisting of apoptosis and mitotically arrested cells, villous atrophy was not observed in the small intestine of intoxicated mice.

In this study, toxicity of autumn crocus was recognized to be consistent with that of colchicine in both guinea pigs and mice. Colchicine has been classified as a microtubule-disrupting drug, and has an ability to arrest mitosis [1, 6, 10]. Cellular injury caused by colchicine tends to distribute in proportion to the tissues which have high activity of cell reproduction [2]. In the present study, the intestinal crypt epithelium was most severely affected by both autumn crocus and colchicine. The

lesions seen in the small intestine were more severe than those seen in the large intestine, suggesting that the toxicity of autumn crocus may be closely associated with the frequency of the cell turnover in the same way as colchicine.

Mice given autumn crocus or colchicine in the present experiment did not develop diarrhea, unlike guinea pigs. Villous atrophy was seen only in intoxicated guinea pigs, whereas the crypt change was seen in both guinea pigs and mice. This suggests that the enterotoxicity of autumn crocus or colchicine has a species-difference, and that the significant factor causing the diarrhea is related to villous atrophy rather than crypt change.

It has been known that the mechanism of disposal of effete enterocytes differs among species [8, 9, 11–13]. In the rat and mouse, effete epithelial cells are wholly exfoliated into the lumen [8, 13]. Apoptotic enterocytes in the guinea pig, monkey, horse and cattle are phagocytized by macrophages which aggregate at the lamina propria of the villous tips, and only a thin cortex of the enterocytes is shed off [8, 9, 11, 12]. With this in mind, one may assume that the guinea pig may have high reproducibility of autumn crocus poisoning of cattle. The present experimental study was carried out according to this hypothesis, and it was then experimentally corroborated. The results obtained suggest that the mechanism of disposal of effete enterocytes may play an important role in diarrhea caused by autumn crocus or colchicine, although the significant factor determining the species-difference of colchicine toxicity remains to be solved. A more detailed further study on the basis of the species-difference and disposal of the effete enterocytes in the villous tips is needed to fully elucidate the mechanism of acute poisoning of autumn crocus and colchicine.

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