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Ninety-day dietary toxicity study of apple polyphenol extracts in Crl: CD (SD) rats

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ABSTRACT

To examine the safety of Dietary Applephenon® (AP) in feed, Crl: CD (SD) rats of each sex were divided into four groups and given diets containing AP at 0%, 1.25%, 2.5%, or 5.0% for 90 days. All rats survived and toxic changes were not observed throughout the study. Body weight and food efficiency in the 5.0% AP group of both sexes were significantly decreased compared with that in controls. These changes were considered to be caused by the physiological effects of AP (including the inhibitory effects on pancreatic lipase activity). Slight hypertrophy in acinar cells in the parotid and submandibular glands appeared in the 2.5% and 5.0% groups. These were suggested not to be toxicological but physiologic adaptive responses to oral stimuli by the lower pH of AP-containing diets. In conclusion, dietary AP in feed, up to a maximum level of 5.0% for 90 days, given to rats did not induce toxicological effects.

1. Introduction

Apples contain several types of polyphenols. These include procyanidin, epicatechin, catechin, *p*-coumaroyl quinic acid, chlorogenic acid, rutin, and phloridzin; the main components of polyphenols are oligomeric procyanidins (Spanos et al., 1990; Lister et al., 1994; Ohnishi-Kameyama et al., 1997; Suárez et al., 1998; Mangas et al., 1999; Shoji et al., 2003). Applephenon® (AP) is a polyphenol extract produced from the juice of unripe apples using column chromatography. It consists mainly of procyanidins, which are composed of epicatechin and catechin (Shoji et al., 2006a).

AP has been reported to have various physiological effects. These include anti-allergic effects (Kanda et al., 1998; Akiyama et al., 2000, 2005; Nakano et al., 2008; Enomoto et al., 2006), lowering effects on serum cholesterol (Nagasako-Akazome et al., 2005), inhibitory effects on post-prandial levels of triglycerides (Nagasako-Akazome et al., 2007) and anti-obesity effects (Osada et al., 2006; Akazome et al., 2005) *in vivo*. Antioxidant effects (Leontowicz et al., 2002; Schaefer et al., 2006), antitumor effects (Lapidot et al., 2002; Gossé et al., 2005; Hibasami et al., 2004; Miura et al., 2008), inhibitory effects on pancreatic lipase activity (Sugiyama et al., 2007) and micellar solubilization *in vitro* (Ogino et al., 2007) have also been suggested. Furthermore, recent research has indicated that procyanidins from apples have sir-2.1-dependent anti-aging effects on *Caenorhabditis elegans* (Sunagawa et al., 2011). Focusing on these effects, AP has been used widely as a dietary supplement or as an antioxidant food additive for various processed foods in Japan. It was generally recognized as safe (GRAS) in 2004 in accordance with regulations set by the Food and Drug Administration (FDA) in the USA.

We have reported on the safety of AP using the Ames test, acute-oral toxicity test, and 90-day subchronic-toxicity test (Shoji et al., 2004). In the latter, AP was administered every day to Crl: CD (SD) rats at 0, 500, 1000 or 2000 mg/kg body weight (BW) by oral gavage. However, the oral toxicity of other food materials and food additives were examined primarily by the 90-day oral feeding test, in which the method of administration is close to the manner of daily intake of food materials in meals (Wren et al., 2002; Yamakoshi et al., 2002; Bentivegna and Whitney, 2002; Takami et al., 2008; Jin et al., 2007; Goldsmith, 2000; Casterton et al., 2009).

Therefore, in the present study, we undertook another 90-day subchronic test to examine further the safety of dietary AP in feeds at 0%, 1.25%, 2.5% and 5.0%.

2. Materials and methods

This study was carried out in accordance with Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives of Japan (1996).

2.1. Test materials and diet

AP (Asahi Food and Healthcare Co. Ltd., Tokyo, Japan) was used. It was prepared from unripe apple juices (*Malus pumila* cv. Fuji) by solid phase extraction with SEPABEADS® SP-70 (Mitsubishi Chemical Corporation, Tokyo, Japan) (Shoji et al., 2003). The composition of AP is shown in Table 1.

Test diets were prepared by mixing 0%, 1.25%, 2.5%, or 5.0% AP to the basal diet (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and all of these were gamma-sterilized. The uniformity and stability of AP in each test diet was confirmed using a published method (Shoji et al., 2006b). These diets were kept in a refrigerator at 4.2–6.5 °C throughout the test period.

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Table 1
Content of polyphenol in AP.[a]

Content	(%)
Procyanidins	
Dimer	11.1[b]
Trimer	12.3[b]
Tetramer	8.7[b]
Pentamer	5.9[b]
Hexamer	4.9[b]
Over heptamer	20.9[b]
Flavan-3-ols	
(+)-Catechin	2.0[c]
(−)-Epicatechin	10.5[c]
Chalcones	
Phloridzin	1.9[c]
Phloretin-2′-xyloglucoside	4.6[c]
Phenolcarboxylic acids	
Chlorogenic acid	8.2[c]
p-Coumaroyl quinic acid	2.6[c]

[a] Each component was identified by NMR and spectral analysis (procyanidins and phloretin-2′-xyloglucoside) or by comparison of chromatograms with authentic standards ((+)-catechin, (−)-epicatechin, phloridzin, and chlorogenic acid).
[b] Analyzed by normal phase-HPLC.
[c] Analyzed by reversed phase-HPLC.

2.2. Animals

Eighty-eight (44 of each sex) 4-week-old Crl: CD (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The general condition of all animals was checked daily for 12 days and gains in BW noted. They underwent ophthalmological examinations in quarantine as well as an acclimatization period before study initiation. Forty healthy rats of each sex were finally selected for the study. Five of the remaining animals were used for the assessment of viral infections and the other three rats were excluded as subjects and killed.

3. Experimental

The present study was carried out by Mitsubishi Chemical Medience Corporation (Tokyo, Japan) in compliance with Good Laboratory Practice (GLP) and in accordance with the guidelines for animal studies of Mitsubishi Chemical Medience Corporation.

Male and female rats (age, 6 weeks; 40 rats of each sex) were maintained individually in stainless-steel cages at 23.1–24.3 °C, relative humidity (55.1–66.8%) and 12-h lighting (07:00–19:00). They were divided into four groups and given feed containing the test material at 0% (control), 1.25%, 2.5%, or 5.0% for 90 days. Drinking water was also provided to the animals *ad libitum*. The initiation day and week of the administration was each set as "day 1" and "week 1", respectively.

The viability of the animals was observed twice daily. BW and food consumption were measured every 7 days from day 1 to day 85, and day 90. Food efficiency (food consumption/weight gain (%)) and the mean intake of test articles (mg/kg BW/day) were calculated from BW and food consumption, respectively. Water consumption was also measured for 24 h from day 87 to day 88. Furthermore, an ophthalmological examination was done on 5 selected animals at the end of the administration period (day 89).

Urinalyses were conducted at week 13 (days 87–88). Animals were put into the metabolism cages individually. Fresh urine samples were collected for a fixed period (07:41–09:22). Subsequently, 24-h urine samples were collected under the fasting (but free-water drinking) condition. These collected samples were stored at −80 °C. Fresh urine samples were used for the measurement of pH, protein, glucose, ketone body, bilirubin, occult blood, and urobilinogen using urinary test strips (Pretest 8aII; Wako Pure Chemical Industries Ltd., Osaka, Japan). The volume (mL/24 h) and color of urine was measured using urine samples collected over 24 h. In addition, samples were used for the measurement of osmotic pressure (osm/kg) by an OSMOMAT 030-D-RS Osmometer (Gonotec, Berlin, Germany) as well as levels of sodium, potassium and chloride (mEq/24 h) using a PVA-αIII system (Analytical Instruments Co., Ltd., Tokyo, Japan). Diets and drinking water were given in the usual way after the collection of fresh urine.

After the 90-day administration period, rats were fasted overnight (17–22 h). Blood samples were collected from the abdominal aorta under anesthesia (pentobarbital sodium, 30 mg/kg bw, i.p.). They were mixed with ethylenediamine tetra-acetic acid (EDTA) and used for hematological tests by employing an ADVIA 120 system (Bayer Diagnostics Manufacturing Ltd., Newbury, UK). The parameters were white blood cells (WBCs), red blood cell (RBCs), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), sickle cell count, platelet count and WBC differential count. The other blood samples were mixed with 3.8% (*w*/*v*) of sodium citrate and centrifuged at 1870*g* for 15 min to collect the plasma. In addition, serum was collected by centrifugal separation (1870*g* for 10 min at room temperature). The plasma was used for the blood coagulation test to obtain the prothrombin time (PT) and activated partial thromboplastin time (APTT) (Sysmex CA-5000; Sysmex Co., Kobe, Japan). Serum samples were assessed using an autoanalyzer (7170; Hitachi, Ltd., Tokyo, Japan) for the following biochemical parameters: total protein (T. protein), total bilirubin (T. bilirubin), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GTP), alkaline phosphatase (ALP), total cholesterol (T. cholesterol), triglycerides, phospholipids, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus (IP), calcium, and magnesium. Measurements for creatine phosphate kinase (CPK) and lactate dehydrogenase (LDH) were made using plasma. Differential serum protein and the ratio of albumin/globulin (A/G) were analyzed using an AES320 system (Olympus Co., Tokyo, Japan). Blood levels of sodium, potassium, and chloride were analyzed by a PVA-αIII system (Analytical Instruments Co., Ltd.).

Subsequently, rats were killed and necropsy findings recorded. The brain, pituitary gland, submandibular glands, thyroid glands, heart, lungs, thymus, liver, spleen, kidneys, adrenal glands, seminal vesicles, prostate gland, testes, ovaries, and the uterus were removed and their weights (absolute weights) recorded. Relative weights (calculated by dividing the organ weight by the final BW on day 91) were also documented.

Samples of the following organs and tissues were removed and fixed in phosphate-buffered 10% formalin for histopathological evaluation: tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, submandibular glands, sublingual glands, parotid glands, liver, pancreas, nasal cavity, trachea, lungs, thymus, submandibular lymph node, mesenteric lymph node, spleen, bone-marrow, heart, aorta, kidney, urinary bladder, prostate, seminal vesicle, ovary, oviduct, uterus, vagina, mammary gland, pituitary gland, thyroid glands, parathyroid gland, adrenal glands, cerebrum, cerebellum, medulla oblongata, spinal cord, sciatic nerve, Harderian gland, Zymbal's gland, musculus biceps femoris, sternum, femur and integument. Eyes and optic nerves were fixed in Davidson's solution. The testes and epididymis were fixed in Bouin's solution. Histopathological tests were carried out in animals in the control group and 5.0% group of each sex. In addition, the submandibular glands in the 2.5% groups and parotid glands in the 1.25% and 2.5% groups of sexes were also investigated.

3.1. Statistical analyses

The following parameters were analyzed using the MiTOX-PPL system (Mitsui Zosen Systems Research Inc., Chiba, Japan): BW, food consumption, food efficiency, water consumption, as well as