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Exploration for unknown substances in rapeseed oil that shorten survival time of stroke-prone spontaneously hypertensive rats Effects of super critical gas extraction fractions

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Abstract

To identify the causative substances for the shortening of survival time by rapeseed (Canola) oil in stroke-prone spontaneously hypertensive rats (SHRSP), SHRSP were fed on a standard chow supplemented with 10 w/w% soybean oil (control), rapeseed oil, one of the fractions of rapeseed oil obtained by super critical gas extraction (SCE) under a pressure of 180-bar or 350-bar, at 40 °C, or the residue from the extraction (with 0.5% NaCl in drinking water). In another series of experiment, SHRSP were fed for 8 weeks on the above-mentioned diets without salt loading and autopsied. Fatty acid compositions in these diets were similar, except in the soybean oil diet, and phytosterol contents were: (diet containing) 180-bar fraction > residue > rapeseed oil > 350-bar fraction > soybean oil. Survival times in the rapeseed oil, 350-bar fraction and residue groups were shorter than, whereas that in the 180-bar fraction was similar to in the soybean oil group. In the 8-week feeding experiment, chronic nephropathy was found frequently in the groups other than the soybean oil group. The heart weights were higher in the rapeseed oil and residue groups. Cerebral necrosis was found in the residue group. Taken together, the followings are concluded, (1) Neither the fatty acid composition, nor the amount of phytosterols in the diets appeared to be decisive in the shortening of life. (2) SCE appeared to produce a safe (180-bar) fraction, though it failed to separate clearly the causative substances into specific fractions. (3) The factors that facilitate the genetic disease of SHRSP appear to exist in rapeseed oil. However, they might not be identical to those responsible for the life-shortening, since there were no findings common across the rapeseed oil, 350-bar and residue groups, which showed similar life-shortening.

Keywords: Super critical gas extraction; Rapeseed (Canola) oil; SHRSP; Survival time; Brain; Kidney

1. Introduction

In 1996, Huang et al. reported the life-shortening effects of some vegetable oils, including rapeseed (Canola) oil, in stroke-prone spontaneously hypertensive rats (SHRSP) when these oils were given supplementarily to a standard rat chow. This life-shortening effect has recently been found to be transmittable from maternal rats to pups (Tatematsu et al., 2004b). The causative factors were supposed to be relatively lipophilic, non-polar and lipase-sensitive, and have no relation to fatty acids or phytosterols (Huang et al., 1997; Miyazaki et al., 1998). On the other hand, Ratnayake et al. (2000a,b) demonstrated that the amount of phytosterols in several vegetable oils, including rapeseed oil, might play a key role in the shortening of survival time in SHRSP by replacing membrane-bound cholesterol or by affecting the

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metabolism of endogenous cholesterol. Naito et al. (2003) also reported that the shortening of survival time by rapeseed oil seems be due to exacerbation of hypertension-related or stroke-related conditions in rats of this strain, and that phytosterols in the oil may be at least one of the causative factors.

While phytosterols may not have been ruled out of the possible causes for the life-shortening effects of rapeseed oil, it should be taken into consideration that the amount of phytosterols in vegetable oils does not necessarily increase in parallel with the life-shortening effects. For example, olive oil, which is not phytosterol-rich, also shortened the survival time of SHRSP (Huang et al., 1997; Ratnayake et al., 2000a). It is, therefore, of interest to make an attempt to identify the causative substances, other than phytosterols or fatty acids, in these oils.

In the present study, as the first step in exploring the causative substances, we used three fractions from rapeseed oil by super critical gas extraction (SCE). SHRSP were fed with diets, each containing soybean oil, rapeseed oil or one of the fractions from rapeseed oil and the onset of stroke-related signs, survival times, and pathological findings in the organs were compared. In addition, scheduled autopsy was carried out in SHRSP fed the same diets for 8 weeks for hematological, blood biochemical and histological examinations.

2. Materials and methods

2.1. Super critical gas extraction (SCE) fractions of rapeseed oil

Three fractions of rapeseed oil were obtained by SCE (Mohri Oil Mill, Co., Ltd., Matsuzaka, Japan). In brief, 13 kg of rapeseed oil was placed in a 500-l extraction chamber where the temperature was kept at 40 °C, and the extraction was carried out over 6 h 30 min under a pressure of 180-bar, and then, for 11 h at 350-bar. Carbon dioxide gas was supplied at a rate of 1.5 kg/min during the extraction. Each extract was separated from the carbon dioxide in a separator chamber at 40 °C and at 60 bar. The amounts of the extracts were 1.3 kg, 9.0 kg and 2.5 kg for the 180-bar fraction, 350-bar fraction and residue, respectively (total yield, 98.6%).

The extraction pressures we chose were just two, the highest pressure of which the apparatus was capable and a pressure that was about half of that (i.e., a middle value), because as we could not predict with certainty which ingredients would be in which extracts at various pressures, we saw no benefit to selecting more than two extraction pressures, and because even if we had selected more than two extraction pressures, the experiments to make fraction-by-fraction determinations of the biological effects at many different extraction pressures would have required sacrificing a huge number of animals without being certain beforehand of the need to do so.

2.2. Preparation of rat diets

A standard powder diet for rats (CE-2, CLEA Japan, Tokyo) was mixed with rapeseed (Canola) oil (Ohta Oil Mill Co., Ltd., Okazaki, Japan) one of the three fractions of rapeseed oil, or soybean oil (Ohta Oil Mill Co., Ltd.) at 10 w/w% (12.5–13.0 w/w% in total fat) and made into pellets. The diet containing soybean oil was considered as the control, because CE-2 diet already contains soybean oil as its major fat ingredient.

2.3. Fatty acid analysis

Fatty acid compositions in the diets were determined using the AOCS (American Oil Chemists Society) Official Method Ce 1b-89. In brief, an aliquot, 1–1.5 g of diet, was mixed with 35–60 mg of heptadecanoic acid, an inner standard, 2 ml of ethanol and 10 ml of 5 mol/l HCl solution. Then, 50 ml of diethyl ether/petroleum ether (1:1) mixture was added to extract fatty acids into the mixture phase in the presence of 8 ml ethanol. This extraction procedure was repeated three times. The diethyl ether/petroleum ether mixture was washed with water and the solvents were vaporized. Fatty acids obtained were saponified by adding 1.5 ml of 0.5 mol/l NaOH ethanol solution, and then, heated to 100 °C for 9 min. Saponified fatty acids were methyl esterified by adding 2 ml of 14% BF3/methanol reagent and heated to 100 °C for 30 min. Three milliliters each of hexane and saturated NaCl solution were added and mixed. Esterified fatty acids obtained were measured by gas chromatography using a GC-17A (Shimadzu, Kyoto, Japan) with Column J&W DB-23, 0.25 mm diameter × 30 m. The oven was kept at 50 °C for 1 min. The temperature was, then, increased to 170 °C at a rate of 10 °C/min, and then, to 210 °C at a rate of 1.2 °C/min. The temperature of the detector and injector was 250 °C. The flow-rate of the carrier gas (helium) was 1.5 ml/min at 60 kPa H_2 and 50 kPa air. Splitless injection was used. The injection volume was 1–2 μl.

2.4. Phytosterol analysis

Phytosterol composition was determined as follows.

An aliquot, 1–2 g of the diet was mixed with 2 ml of ethanol and 10 ml of 5 mol/l HCl solution. Fatty acids were removed by saponification by adding 50 ml of 1 mol/l KOH/ethanol solution. One hundred and fifty milliliters of water and 100 ml of diethyl ether were added to obtain non-saponified substances in the organic solvent. This extraction procedure was repeated three times. The collected diethyl ether phase was washed with water and desiccated. An adequate amount of hexane was added, and the mixture was placed in a column SEP PAK Silica Cartridges (Waters Japan Co., Ltd., Tokyo, Japan) and extracted with 25 ml of diethyl ether/hexane 20:80. The elute, to which was added 0.5 mg 5α-cholestane, was evaporated. An adequate amount of hexane was again added, and the mixture was injected into a gas chromatograph, GL Science GC-353 with Column J&W DB-1, 0.25 mm diameter × 15 m (GL Science, Tokyo, Japan). The temperature of the column was increased from 240 °C to 270 °C at a rate of 3 °C/min. Temperature of the detector and injector was 290 °C. The flow-rate of the carrier gas (helium) was 2.3 ml/min at 110 kPa H_2 and 250 kPa air. Splitting ratio was 1:30. The injection volume was 1–2 μl.

2.5. Animal maintenance and experiments

2.5.1. Survival time experiment

One hundred male SHRSP (Izm), 4 weeks old {Disease Model Cooperative Research Association (Kyoto)} were acclimatized for 1 week, and then divided into five groups of 20 animals each. The animals of each group fed a standard rat chow to which was added 10 w/w% soybean oil, rapeseed oil, or one of the three SCE fractions of rapeseed oil, that is, the 180-bar fraction, the 350-bar fraction or the residue. The animals were allowed free access to the diet and drinking water, which was prepared by dissolving 0.5 w/w% NaCl in distilled and de-ionized water. NaCl-loading was used to accelerate the progress of hypertension (Sapirstein et al., 1950) for amplify any differences in effects of the treatments. All the animals were observed at least once a day for general condition. The following symptoms were regarded as signs of stroke: exophthalmus, hyperirritability, hyperkinesia, hyporesponsiveness, motion disturbance, tremor, convulsion, limb paralysis or sudden death (Okamoto et al., 1974; Nagaoka et al., 1976). The animals were weighed on the day before starting the administration, and thereafter, once a week. Food intake and water intake were also measured once a week.

Postmortem examinations were carried out on all the animals as soon as possible after they were found dead. The brain, heart, lungs, spleen, liver, adrenal glands and kidneys were removed, macroscopically observed and weighed. Since a major purpose of the study was to examine survival time, most of the animals could not be examined immediately after death. Histological examination was, thus, carried out in only a few animals, in which autopsy could be carried out immediately after death: in three animals of the residue group and in two animals each of other groups.