JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 5659−5663 **5659**

Nephrocalcinosis Formation by Soy Isoflavones in Male Rats

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Isoflavones (IFs), found in the form of both aglycones and glucosides in soybean foods, induce weak estrogenic activities. Although IFs have a number of health benefits, it was previously reported that IFs cause nephrocalcinosis (NC) in the kidney of male Fischer 344 (F344) rats. The present study aims to elucidate the safety of IFs by focusing on IF-induced NC formation in rats. Fermented soybean extract (FSE) containing 420 mg/g isoflavone aglycones was orally administered to male F344 and Sprague−Dawley (SD) rats for 28 days. FSE induced NC formation in the kidney of F344 rats, but not in SD rats. However, absorption of IFs did not differ between F344 and SD rats. NC formation and its severity of FSE were histologically compared with those of soybean extract (SE) containing 518 mg/g isoflavone glucosides in F344 rats. There were no differences in the number of NC formations and the extent of calcium deposit between FSE and SE groups. To examine the dose effect of FSE on NC formation, doses of 20, 140, or 1000 mg/kg FSE were administered to F344 rats for 90 days. NC formation was observed in the 140 and 1000 mg/kg groups. These results indicated that a high dose of oral administration of IFs induced NC formation depending on the strain of rat.

KEYWORDS: Isoflavones; nephrocalcinosis; calcium deposit; F344 rats

INTRODUCTION

Isoflavones (IFs), naturally occurring as plant chemicals, provide a number of health benefits, such as reducing the risk of cancer, attenuation of bone loss, and relief of menopausal symptoms (*1*, *2*). It has been reported that IFs are mainly found in soy-based foods (*3*). Soybeans contain IFs as glucosides, and traditional Japanese foods such as soy sauce and miso contain not only glucosides but also aglycones (*3*, *4*).

To define the distinction between isoflavone aglycones and glucosides, comparative studies of weight gain (*5*, *6*) and pharmacokinetics of absorption (*7*−*11*) have been conducted in rats and human. Isoflavone aglycones are absorbed from the stomach and small intestine directly (*7*). On the other hand, isoflavone glucosides are converted to isoflavone aglycones by gut microflora or gut glucosidases and then absorbed from the intestines (*8*). It has been reported that isoflavone aglycones are absorbed more quickly than isoflavone glucosides in people of Japanese origin (*9*), although both isoflavone aglycones and glucosides are similarly absorbed in Americans (*10*).

Toxicological studies of genistein, a soy isoflavone extract and a red clover isoflavone extract, have reported that the administration of IFs to F344 rats causes histopathological changes such as an increase of apoptotic cells and epithelial detachment and hyperplasia in the ovary, epididymus, and ventral prostate (*12*−*14*).

We have previously evaluated the safety of fermented soybean extract (FSE) that contains 420 mg/g isoflavone aglycones in acute and subchronic toxicity studies (*15*) and a genotoxicity study (*16*). The subchronic toxicity study showed that oral administration of FSE once daily for 90 days caused nephrocalcinosis (NC) in the kidney of male F344 rats. We also conducted a comparative feeding study of isoflavone aglycones and glucosides, in which these IFs induced NC formation in F344 rats by oral administration (*17*). These studies suggested the possibility of IF administration causing NC formation in rats; however, no other detailed study has been conducted.

NC in rats is a pathological condition with calcified deposits along the entire corticomedullary junction of the kidney (*18*). Strain (*19*), diet (*20*), and age (*21*) have been reported as etiologic factors of NC. In particular, sex is considered to be the principal factor of NC etiology (*22*). The rats treated with estorone following ovariectomy revealed an increase in the degree of NC (*23*), suggesting that sex hormones, especially estrogen, appear to induce NC. It is well-known that IFs exert a weak estrogenic activity. However, there is little information regarding the influence of IFs on NC formation in rats. In addition, the mechanism underlying NC formation induced by IFs is not fully understood.

Recently, IFs have been used as functional foods and dietary supplements due to their health benefits. Therefore, it is

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10.1021/jf060295l CCC: $33.50
Published on Web 06/29/2006

Table 1. Composition of FSE and SE That Were Tested in the Present Study[a]

composition	FSE	SE
isoflavone aglycones		
genistein	230.0	2.9
daidzein	190.0	3.7
glycitein	ND[b]	1.4
isoflavone glucosides		
genistin	ND	268.0
daidzin	ND	182.0
glycitin	ND	31.4
6″-*O*-acetylgenistin	ND	17.8
6″-*O*-acetyldaidzin	ND	18.5
6″-*O*-acetylglycitin	ND	ND
6″-*O*-malonylgenistin	ND	ND
6″-*O*-malonyldaidzin	ND	ND
6″-*O*-malonylglycitin	ND	ND
protein	200.0	71.0
carbohydrate	165.0	336.5
fat	135.0	11.8
moisture	29.0	26.0
ash	29.0	29.0
fiber	22.0	ND

[a] Number = mg/g. [b] Not detectable.

indispensable to detail the safety of IFs. The aim of the present study is to elucidate the influence of IFs with regard to NC formation.

In the present study, we first administered FSE containing isoflavone aglycones to male F344 and Sprague−Dawley (SD) rats to determine whether different strains of rats affect IF-induced NC formation. To compare the responses of F344 and SD rats toward estrogenic compounds, some studies have been conducted (*24*, *25*). However, these have been mainly focused on the reproductive tracts, and little has been reported about NC formation in the kidneys. Second, we compared the NC formation of isoflavone aglycones to that of glucosides in detail by counting and measuring the number and area of calcium deposits per kidney. The findings will provide useful knowledge of NC formation and the safety of IFs.

MATERIALS AND METHODS

Chemicals. FSE was made by Kikkoman Co., Ltd. (Chiba, Japan). FSE was extracted and purified by ethanol/water from fermented soybeans. Soybean extract (SE) was purchased from ADM Co. (Decatur, IL). The composition of FSE and SE is shown in **Table 1**. A 0.5% sodium carboxymethyl cellulose (CMC-Na; Wako Pure Chemical, Osaka, Japan) solution was used as a vehicle for suspended FSE and SE. Daidzein, genistein, and H-1 sulfatase were purchased from Sigma Chemical Co. (St. Louis, MO). Glycitein was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were of HPLC grade.

Animals. Male F344 and SD rats, aged 4 weeks old, were purchased from Charles River Japan Inc. (Kanagawa, Japan). NC formation was examined in male F344 rats in comparison with male SD rats because of spontaneous NC formation in female F344 rats (*23*). They were fed a normal diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. Animals were allocated to groups using a randomized procedure based on body weight. During the experiments, the rats received human care consistent with the institutional guidelines of Kikkoman Co., Ltd.

Administration of FSE and SE. FSE was administered once daily at 1000 mg/kg ($n = 10$ per group) to F344 and SD rats by oral gavage for 28 days to examine NC formation. In the next experiment, NC formation by FSE was compared to that of SE, in which FSE was orally administered once daily at 400 or 800 mg/kg and SE was also administered at 613 or 1226 mg/kg in the same manner to F344 rats ($n = 10$ per group) for 28 days. Furthermore, a dose of 20, 140, or 1000 mg/kg of FSE was orally administered once daily to F344 rats ($n = 15$ per group) for 90 days to examine the subchronic effect on NC formation. The control group received 0.5% CMC-Na solution in the same manner. At the end of experiment, rats were euthanatized 1 h after the administration under the anesthesia of sodium phenobarbital.

Histological Examination. Immediately after euthanasia, the left kidney was freshly removed and cut longitudinally at the median. Each kidney was fixed in neutral buffered formalin for 1 week at room temperature and routinely embedded in paraffin. The sections in 2 μm were deparaffinized and stained with hematoxylin and eosin. Some sections were also stained according to the von Kossa PAS method (*26*) to detect the presence of calcium salts. The number of NC formatons per section was also counted under light microscopy. The area of calcium deposit in sections was measured using the Color Image Analyzer (SP500F, Olympus Co., Ltd., Tokyo, Japan).

Determination of Total Renal Calcium. The left kidney was freshly removed and dehydrated in an incubator at 105 °C for 10 days. The dried kidneys were dissolved in 1 N HNO_3. The concentration of Ca^{2+} (μg/mL) was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES, Optima 3000, Perkin-Elmer, Fremont, CA).

Determination and Measurement of Isoflavones in Plasma. The blood was collected to quantify isoflavone concentration in the plasma of F344 and SD rats that were daily administered FSE at 1000 mg/kg for 28 days. The concentration of isoflavones was measured according to a method described in detail elsewhere (*27*). An aliquot (0.2 mL) of plasma was mixed with 50 μL of H-1 type sulfatase and 0.2 mL of buffer (0.1 M sodium acetate). This mixture was incubated at 37 °C for 1 h in a shaking water bath. Released genistein and daidzein from conjugated form were extracted with 0.9 mL of MeOH/acetic acid (95:5, v/v) with sonication and vortexing and centrifuged at 5000*g* for 5 min at 4 °C. The supernatant was diluted to double its quantity with 100 μmol/mL lithium acetate in water, and diluted samples were used for HPLC.

Analysis was performed using the CoulArray model 5600 HPLC detection system (ESA Inc., Chelmsford, MA) with an Inertsil ODS-3 column (150 × 3.0 mm i.d., 3 μm, GL Sciences Inc., Tokyo, Japan). The system was controlled using the CoulArray software on a Pentium-based computer. Mobile phase A consisted of 20% MeOH (v/v) in 50 mM sodium acetate buffer (pH 4.8). Mobile phase B consisted of 40% MeOH (v/v) and 20% acetonitrile (v/v) in 50 mM sodium acetate buffer (pH 4.8). The gradient consisted of initial conditions of 20% mobile phase B and a linear increase to 100% B over 25 min, followed by a 5-min hold at initial conditions. The flow rate was kept constant at 0.4 mL. The detector settings were 440 mV.

Statistical Analysis. Statistical differences between the groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparisons test. Statistical analyses were tested using SPSS for Windows. A value of <0.05 denoted the presence of a significant difference.

RESULTS

Comparative NC Formation in Different Strains of Rats. NC formation in the kidney section of F344 rats that were daily administered FSE at 1000 mg/kg for 28 days was confirmed to be a calcium deposit by using the von Kossa staining method (**Figure 1**). NC resulted in the accumulation of calcium phosphate precipitates in the outer stripe of the outer medulla of the kidney. The calcium deposits at the junction of the outer and inner stripes were primarily located in terminal segments of proximal tubules (**Figure 2**). A high incidence of NC by FSE administration was observed in male F344 rats (**Table 2**). However, FSE administration to male SD rats did not induce any NC. NC was not observed in control groups of either F344 or SD rats.

The total renal calcium concentration in the kidneys of the control group was significantly higher in F344 rats than in SD rats (**Table 2**). By the administration of 1000 mg/kg FSE, the calcium levels significantly increased in SD rats. However, the

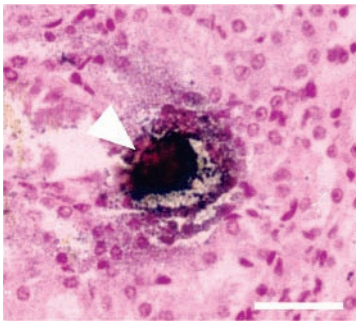

Figure 1. NC in the kidney section of F344 rat. The section was stained according to the von Kossa method. NC (arrowhead) is detected as a calcium deposit. Bar = 40 μm.

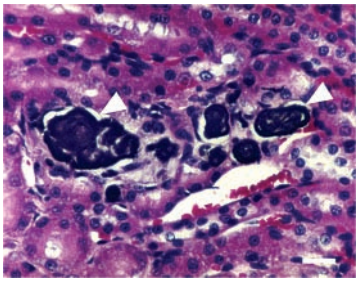

Figure 2. Localization of calcium deposit in the kidneys of F344 rats. Calcium deposits (arrowheads) primarily appear in terminal segments of proximal tubules that lie in the outer stripe of the outer medulla. Bar = 10 μm.

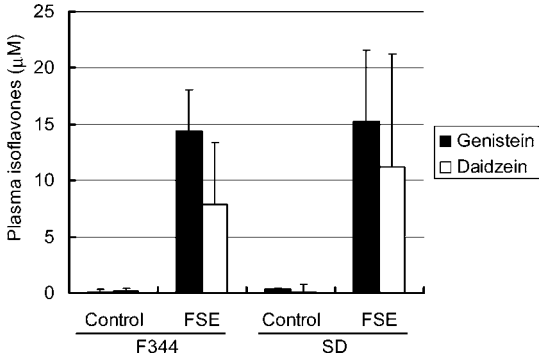

Figure 3. Plasma concentrations of IFs in F344 and SD rats. Data are means ± SD of 10 animals in each group.

Table 2. Incidence of NC and Renal Calcium Concentration in F344 and SD Rats after FSE Administration[a]

	F344		SD	
	control	FSE	control	FSE
incidence	0	90	0	0
Ca conc[b]	511 ± 161AB	551 ± 38A	274 ± 31C	386 ± 15B

[a] FSE was administered at 1000 mg/kg for 28 days. [b] Calcium concentrations are presented as means ± SD. Means with different letters are significantly different ($p < 0.05$). Renal calcium concentration = μg/g.

level in the FSE group of SD rats was lower than that in the control group of F344 rats.

Plasma concentration of IFs following FSE administration is shown in **Figure 3**. In FSE groups, substantial genistein and daidzein concentrations were detected, but there were no significant differences between F344 rats and SD rats. The concentrations of their IFs were slightly detected in both control groups of F344 and SD rats.

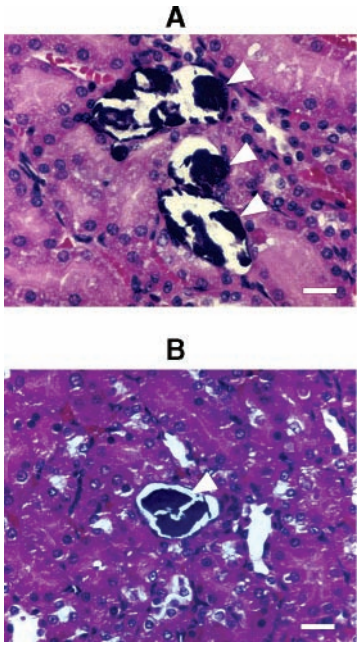

Figure 4. Comparative development of calcium deposit in the kidney of F344 rats administered FSE (800 mg/kg) and SE (1226 mg/kg) for 28 days. NC formation (arrowheads) and its localization by FSE administration (**A**) are almost the same compared with SE administration (**B**). Bar = 10 μm.

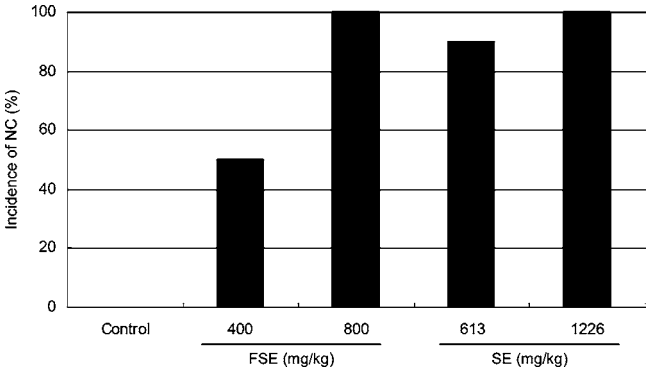

Figure 5. Incidence of NC formation in F344 rats administered different doses of FSE and SE for 28 days. Incidence of NC was calculated by histopathological examination.

Influence of FSE and SE on NC Formation. In male F344 rats, FSE and SE administration similarly induced NC in the kidney (**Figure 4**). The distribution of NC in the SE group was observed in the outer stripe of the outer medulla of the kidney and did not differ from that of the FSE group. A high incidence of NC was observed in the FSE group of 400 mg/kg administration and in the SE group of 613 mg/kg administration (**Figure 5**). All rats formed NC by an 800 mg/kg administration of FSE and a 1226 mg/kg administration of SE, respectively. On the other hand, no NC formation was found in the control group.

The numbers of calcium deposits in the kidney section of male F344 rats were not significantly different between FSE and SE groups, although the number tended to increase with the accompanying dose of FSE and SE administration (**Table 3**). The areas of the calcium deposits varied widely and were 220.3 ± 531.7 μm^2 (400 mg/kg FSE, mean ± SD) and 1172.5 ± 2695.5 μm^2 (800 mg/kg FSE) in FSE groups and 1717.1 ± 1382.9 μm^2 (613 mg/kg SE) and 2337.5 ± 1666.1 μm^2 (1226 mg/kg SE) in SE groups. The values of the calcium deposit areas were larger with the increased doses in FSE and SE

Table 3. Number of Calcium Deposits per Kidney Section in F344 Rats[a]

		FSE (mg/kg)					SE (mg/kg)	
test period	control	20	140	400	800	1000	613	1226
28 days	0 ± 0			2.3 ± 0.9	5.0 ± 3.5		4.3 ± 3.7	6.0 ± 3.9
90 days	0 ± 0	0 ± 0	2.0 ± 1.0A			7.7 ± 4.7B		

[a] Values are presented as means ± SD. Means in the same row with different letters are significantly different ($p < 0.05$).

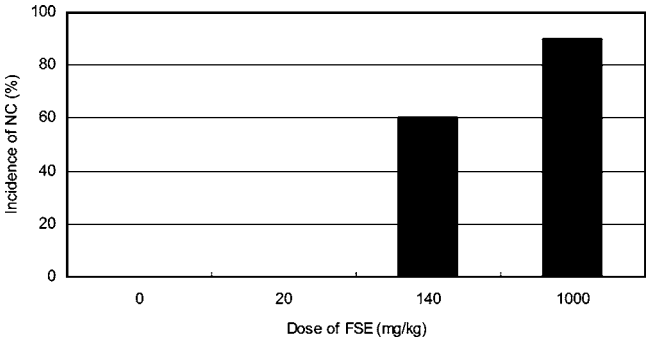

Figure 6. Incidence of NC formation in F344 rats administered different doses of FSE for 90 days. Incidence of NC was calculated by histopathological examination.

groups. However, there were no significant differences between either group.

Subchronic Influence of FSE on NC Formation. After FSE administration for 90 days in male F344 rats, NC was observed in 60% of the 140 mg/kg group and in 90% of the 1000 mg/kg group (**Figure 6**). Administration of 20 mg/kg of FSE did not induce any NC. The distribution of calcium deposit in the kidney did not differ from that of FSE administration for 28 days. The number of calcium deposits significantly increased accompanying the dose of FSE (**Table 3**). The area of calcium deposit in the 1000 mg/kg group was 2660.7 ± 1747.3 μm^2, which was significantly larger than the area of 344.9 ± 1561.4 μm^2 in the 140 mg/kg group ($p < 0.05$).

DISCUSSION

In the present study, we first detailed the influence of FSE on NC formation in F344 rats. NC formation by oral FSE administration at 1000 mg/kg was found primarily at the junction of the outer and inner stripes of the outer medulla, indicating that the findings are consistent with those of Geary, who was first to report the participation of estrogen-type sex hormone in NC development (*22*). Iwata et al. have reported that spontaneous calcium deposits in F344 female rats are not observed in the kidney of ovariectomized female rats, but they are observed in ovariectomized rats treated with estorone, the precursor of estrogen (*23*). They have concluded that estrogen plays a role in the calcium deposits in female F344 rats. Ritskes-Hoitinga et al. have indicated that estrogen elevates plasma concentration of 1,25-$(OH)_2$-vitamin D, which stimulates intestinal calcium (Ca) and phosphorus (P) absorption and tubular reabsorption of these minerals in the kidney. Stimulation of Ca and P (re)-absorption enhances the risk of precipitation of calcium phosphates either intratubularly or intracellularly (*18*). Delclos et al. have reported that exposure to genistein in pups after weaning causes NC (*12*). They have concluded that NC formation observed in pups is due to the estrogenic activity of genistein. In the present study, it is considered that FSE shows estrogenic activity, stimulates Ca and P (re)absorption, and enhances the risk of NC in F344 rats.

Concerning the strains of rats, oral administration of FSE induced NC formation in F344 rats, but not in SD rats. Several researchers have reported rat strain as one of the etiological factors of NC (*28*, *29*). F344 rats are more sensitive to the estrogenic action of diethylstilbestrol, an estrogenic reference compound, than the SD rats (*25*). F344 and SD rats exhibit differences in vaginal responses to bisphenol A, which behaves as a weak estrogen in classic bioassays (*24*, *25*). These reports show that F344 rats are highly sensitive to xenoestrogen compared to SD rats; however, the underlying cause(s) of difference in strain sensitivity to xenoestrogen is (are) currently under investigation. It is suggested that strain differences in the present study on IF-induced NC formation relate to the sensitivity of each strain to the estrogenic activity of IFs. Blood concentration of IFs after FSE administration indicated that there was no difference in genistein and daidzein absorption between F344 and SD rats. On the other hand, the basal level of renal calcium in F344 rats is much higher than that in SD rats, and the renal calcium level was elevated in both F344 and SD rats by FSE administration. Maeda et al. have reported that the renal calcium level in F344 rats is higher than that in SD rats and in Wistar and Donryu rats (*29*). It is considered that a higher level of renal calcium in male F344 rats is one factor of liability in inducing NC formation.

A number of dietary variables are involved in the development of NC, and a low molar ratio of dietary Ca:P is an important contributing factor to NC formation (*30*). In general, a dietary Ca:P ratio of <1 is nephrocalcinogenic in female rats, whereas a ratio of >1.4 prevents nephrocalcinosis (*18*). In the present study, the dietary Ca:P ratio in FSE and SE groups did not fall below 1 (1.24 and 1.25 in 800 mg/kg FSE and 1226 mg/kg SE groups, respectively), suggesting that the dietary Ca:P ratio is not irrelevant to the development of NC.

FSE and SE similarly induced NC formation in the kidneys of F344 rats. The oral dose of FSE as aglycone form was 168 mg/kg (genistein, 92 mg/kg; daidzein, 76 mg/kg) in the 400 mg/kg FSE group, and the dose of SE as the glucoside form was 294 mg/kg (genistin, 164 mg/kg; daidzin, 111 mg/kg; glycitin, 19 mg/kg) in the 613 mg/kg SE group. According to the data of their compounds, there is no difference in IF intake as aglycones between the 400 mg/kg FSE group and the 613 mg/kg SE group, indicating that the intake of IFs as aglycones of FSE was comparable to that as glucosides of SE. The oral administration of FSE or SE for 28 days induced NC in F344 rats; however, there were no significant differences in the area of calcium deposit between the FSE and SE groups. King et al. have reported that genistein is absorbed more rapidly than genistin at 2 h, but there are no significant differences at 8 h or later after the administration (*8*). Their findings suggest that the plasma concentrations of IFs throughout 28 days do not differ in the FSE and SE groups. Taken together, it is considered that isoflavone glucosides induce NC formation in the same manner as those of isoflavone aglycones in male F344 rats.

When a different dose of FSE was orally administered for 90 days, the number of calcium deposits increased and a further area of calcium deposit enlarged accompanying the dose of FSE.

The incidence of NC was detected at a dose of 140 mg/kg, and not at 20 mg/kg. It is likely that NC incidence depends on the increased dose of IFs.

McClain et al. reported that the administration of genistein to Wistar rats and dogs for 52 weeks at a dose of 500 mg/kg/day did not induce NC formation (*31*, *32*). The dose of IFs in their study is much higher than that of our study. Besides, the test period is also much longer than that in our study. These findings also suggest that NC formation by IFs depends on the animal species and strain of rats.

In conclusion, we first detailed NC formation by FSE oral administration in male F344 rats. NC formation depended on the dose of IFs and the strain of rats. The dose of IFs that induced NC formation in rats was much larger than the daily intake of humans. Although these findings provide us with knowledge about the safety of IFs, further studies are needed to clear the mechanism of IF-induced NC formation.

ABBREVIATIONS USED

IFs, isoflavones; F344, Fischer 344; SD, Sprague−Dawley; NC, nephrocalcinosis; FSE, fermented soybean extract; SE, soybean extract.

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Received for review February 1, 2006. Revised manuscript received May 1, 2006. Accepted May 23, 2006.

JF060295L