Safe Dosage of Caprine CSN1S2 Protein from Fresh Local Goat Milk for Rats Evaluated by Acute and Sub-Chronic Toxicity Testing

Fatchiyah[1,5], Bambang Setiawan[2], Nia Kania[3], Takeshi Ohta[4], Vita Agustina[5] & Suharjono[5]

[1]Center Research for Smart Molecule of Natural Genetic Resources, Brawijaya University, Jl. Veteran, Malang, 65145, East Java, Indonesia.
[2]Center Research for Osteoporosis, and Department of Medical Chemistry and Biochemistry, Medical Faculty, Lambung Mangkurat University, Banjarmasin, 70712, South Kalimantan, Indonesia.
[3]Department of Pathology, Ulin General Hospital, Medical Faculty, Lambung Mangkurat University, Banjarmasin, 70233, South Kalimantan, Indonesia.
[4]Biological/Pharmacological Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-1 Murasaki-cho, Takatsuki, Osaka 569-1125, Japan.
[5]Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, 65145, East Java, Indonesia.
E-mail: fatchiya@ub.ac.id

Abstract. Caprine milk CSN1S2 protein contains bioactive peptides that have a specific function in regulating the metabolic homeostasis mechanism between different organs. However, the safe dosage of goat milk consumption has not been established previously. The aim of this study was to evaluate the safe dosage of caprine CSN1S2 protein from fresh local goat milk using acute and repeated doses (for 4 weeks) in oral toxicity studies on three month old normal Wistar rats. The CSN1S2 protein was isolated from fresh goat milk. This casein protein administered in a single oral gavage dose to 5 rat groups at a daily dose of 0, 500, 1000, 2000, 4000 mg/kg BW for acute and 28-day sub-chronic toxicity testing of rats of both sexes. The result showed no evident mortality or abnormal effects. Data analysis of body weight, water and food intake, blood biochemical parameters, clinical observation and organ weight revealed no significant differences between all groups for both sexes. The weights of some organs showed differences between groups, but these values were within the normal weight ranges. The conclusion is that caprine CSN1S2 from fresh milk is safe for food consumption because there was no indication of toxicity, no mortality, and no hematological and clinical chemistry changes.

Keywords: *acute; caprine CSN1S2; goat milk; sub-chronic; toxicity test.*

1 Introduction

Milk protein consists of alpha-S1 and alpha-S2 casein, and beta- and kappa-casein. There are bioactive peptides that are able to reduce the risk of diseases

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or to enhance physiological functions as functional foods and/or nutraceuticals [1,2]. Our previous study identified eight bioactive peptide residues in alpha-S2 casein of goat (caprine) milk with a specific function [3]. Whole and electrophoretic casein fractions of goat milk have been shown to exhibit different biological activities. Goat milk protein is provided as healthy nutrition and as a therapeutic agent to control abnormal or disease conditions through some of its biologically active peptide residues [4]. Alpha-S2 casein (CSN1S2) protein of goat milk has a biological function due to its anti-oxidant, anti-osteoporotic, anti-inflammatory activities, or as an immunomodulatory agent to prevent immune system failure and homeostasis of metabolic mechanisms, respectively [4-6]. Another study explained that goat milk supplementation was also able to protect and reduce the severity of carcinogenesis in hepatocarcinogenesis-induced rats [7].

An important requirement for toxicological experiments on animals is the ability to estimate the effects of the treatment on specific organs. Indicators are a body and organ weight, and histopathological, hematological and biochemical blood parameters. The most sensitive indicator of the effects of experimental treatment with casein protein are significant differences in organ weight between treated and control animals in the absence of any morphological changes. The standard for toxicological evaluation of safety in pre-clinical models can be applied [8]. Biochemical indicators monitoring animal organs such as the liver and kidneys can identify biological markers of tissue damage. The assessment of various enzyme activities in the tissues and body fluids may play a significant role in measuring the effects of molecular compounds related to disease investigation [9,10]. The blood is an analytical subject that is an important indicator of pathological and physiological states in humans and animals. The differentiation of body and organ weight within normal ranges is not associated with variations of hematological parameters causing a lack of clear-dose response [11].

Our previous study also showed that doses of goat milk casein protein at 500, 1000, 1500 and 2000 mg/kg body weight did not result in abnormal microstructure and mineral contents after a month of daily consumption [12]. Although the biological activity of caprine CSN1S2 protein from fresh milk has been revealed, there are no studies available that examined the toxicity of caprine CSN1S2 protein from fresh milk. Therefore, this study aimed to evaluate acute and 28-day sub-chronic toxicity studies of caprine CSN1S2 protein from fresh goat milk in normal Wistar rats.

2 Materials and Methods

2.1 Animal Experiments

In this study, we used 25 male and 25 female, 8-week old Wistar rats, obtained from the Experimental Animal Laboratory, UPT LPPT, Universitas Gadjah Mada, Yogyakarta. The animals were acclimatized for a week in the Experimental Animal Laboratory, Biosains Institute, at Universitas Brawijaya, Malang (conditions prior to experimental manipulation). The rats used as experimental animals were individually housed in polycarbonate metabolic cages and room conditions were maintained (temperature 25 °C, 60% humidity and light-dark cycle 12 hours). The animals had free access to a standard commercial pellet diet and water ad libitum. The conditions and handling of all animals were approved by the Brawijaya University ethical research committee (Ethical Clearance Certificate No. 205-KEP-UB).

2.2 Preparation of Caprine CSN1S2 Protein of Fresh Goat Milk

Caprine Alpha-S2 casein protein was isolated from 250 ml of fresh milk samples from local Ethawah goats, which had been heated to a temperature of 40 °C using a water bath. Glacial acetic acid was added to the milk while stirring until casein precipitate was formed. The caprine Alpha-S2 casein protein was purified by slightly modified standard method [3]. The protein precipitation was then separated by filtration through a nylon mesh [3,5]. The protein content was measured using a Nano-Drop UV-Vis spectrophotometer, after which the protein was stored at -20 °C.

2.3 Administration of Caprine CSN1S2 Protein from Fresh Milk for Acute Toxicity Study

Fifty rats (25 males, 25 females) were divided into five groups (5 males and 5 females in each group): control (untreated) rats 0 mg/kg BW (AMC0/AFC0), and rats treated with CSN1S2 protein at doses of 500 mg/kg BW (AMC500/AFC500), 1000 mg/kg BW (AMC1000/AFC1000), 2000 mg/kg BW (AMC2000/AFC2000), and 4000 mg/kg BW (AMC4000/AFC4000), respectively. The rats were fasted overnight before treatment. The caprine CSN1S2 protein from fresh milk was orally administered at proper doses (as specified below) daily for 14 days, and the control group was given the same volume of vehicle [12]. Single oral gavage doses of caprine CSN1S2 protein from fresh milk (500, 1000, 2000, and 4000 mg/kg BW) were dissolved in d-water according to the previous study, with dose modification [13]. The treated groups were administered with casein in individual cages based on the individual animal body weight obtained on the day of dosing. Symptoms of toxicity, behavioral changes, mortality, and weight changes were monitored on

a daily basis. Finally, the rats were weighed, euthanized, and necropsied on day 15. Mortality and clinical symptoms were observed in a sustainable manner in 0, 1, 2, 3, 4, 5, and 6 hours after dosing on the first day and once per day from day 2 into day 15. The animals were weighed before being dosed (day 1) and after dosing (day 2, 4, 8, and 15) using electronic scales (Sartorius Co., Germany). At the end of each treatment, the rats were given sodium pentobarbital anesthesia (intraperitoneal injection) and their blood was collected from the carotid arteries in heparized and nonheparinized tubes for hematological and biochemical examination of the serum. After collection of the blood, the internal organs were isolated and weighed to determine the relative organ weight and to know the gross lesions.

2.4 Sub-chronic Toxicity Inspection for 28 Days

A study of 28-day sub-chronic toxicity was done according to the Organization for Economic Cooperation and Development (OECD) Guideline 425 adopted on the 3rd of October 2008 and the institutional animal ethics guideline [14]. The casein protein from goat milk was orally administered at proper doses, i.e. 0 mg/kg BW, 500 mg/kg BW, 1000 mg/kg BW, and 2000 mg/kg daily for 28 days, while the control group was given the same volume of vehicle. The rats were then randomly divided into four groups, each group consisting of 6 males (M) and 6 females (F), with a total of 48 rats. Mortality and clinical signs were recorded once per day during the study period using the Path/Tox program (Ver 4.2.2). The experimental animals were weighed on the day of arrival, randomly on the first day of treatment dose, then once a week and at the end of the experiment. External eye examination was done during the pretest period. External and fundus examination were performed using a binocular indirect opthalmoscope (Vantage Plus Digital, Keeler Ltd., UK). At the end of each treatment, the rats were given sodium pentobartial anesthesia (intra-peritoneal injection) and blood was collected from the carotid arteries in heparized and non-heparinized tubes for hematological and biochemical examination of the serum. After collection of the blood, the internal organs were isolated and weighed to determine the relative organ weight and know the gross lesions.

2.5 Hematology and Serum Biochemistry

All test animals were fasted overnight before necroscopy and taking blood. The animals were then given isoflurane anesthesia via inhalation, blood samples were taken before the animals were killed by exsanguination of the aorta. Blood samples were collected in tubes containing EDTA-2K and the complete blood cell count was obtained. The blood cells were measured using a ADVIA 120 Hematology System (Bayer, USA). Prothrombin time (PT) and activated-partial-thromboplastin time (APTT) were analyzed. The blood samples were treated with 3.2% sodium citrate using a Beckman ACL 9000 coagulation

analyzer. Blood samples were collected from the vena cava posterior from all experimental animals for the biochemical and hematological examination, which was conducted according to the OECD Guidelines [13,14]. Serum samples were prepared after centrifugation of the blood and analyzed using an NEO 200FR Toshiba (Toshiba Co., Japan). The biochemical parameters measured were: aspartate aminotransferase (AST, IU/dL), alanine aminotransferase (ALT, IU/dL), alkaline phosphatase (ALP, IU/dL), blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), glucose (mg/dL), total cholesterol (TCHO, mg/dL), total protein (g/dL), triglyceride (TG, mg/dL), uric acid (mg/dL), and bilirubin (mg/dL). The hematological parameters measured were: white blood cells (WBC) ($x10^3/\mu L$), platelet ($x10^3/\mu L$), red blood cell (RBC) ($x10^6/\mu L$), hemoglobin (g/dL), hematocrit (%), mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin (MCH) (pg), and mean corpuscular hemoglobin concentration (MCHC) (g/dL).

2.6 Organ Weight Measurement

The absolute organ weights and relative organ weights (organ/body weight) were calculated for all organs from all the experimental animals that were killed. Absolute organ weights (g) were measured, including the brain, heart, liver, spleen, kidneys, thymus, lungs, adrenals, testes, ovaries, stomach and intestines.

2.7 Statistical Analysis

Data are presented as mean ± SD and differences between groups were analyzed using an ANOVA test with SPSS 16.0 software. A probability value of $P < 0.05$ was considered statistically significant and $P < 0.05$ for all data analyses.

3 Results

3.1 Characterization of Rats' Body Weight, Water & Food Intake and Respiratory Rate

The caprine casein protein was isolated from fresh goat milk as proper. In this 14-day acute toxicity test, the effect of goat milk casein at different concentrations on rats was observed. The rats' body weight gain developed normally in both sexes. Statistical analysis for all treatment groups showed that there was no significant difference between groups ($P > 0.05$) (Figure 1).

Water and food intake of the rats treated with CSN1S2 protein goat milk in the 14-day acute toxicity test in all groups was slightly up and down. Food intake at the doses of 1000 and 2000 mg/kg BW was significantly lower compared to control and at the dose of 500 mg/kg BW. At the dose of 4000 mg/kg BW it

was significantly higher compared to the dose of 1000 mg/kg BW. However, it is assumed that food consumption was still within the common daily range. Further, in order to identify respiratory distress, according to common signs shown by small animals, the respiratory rate was measured. The data for all animal groups did not show any symptoms related to the respiratory distress syndrome.

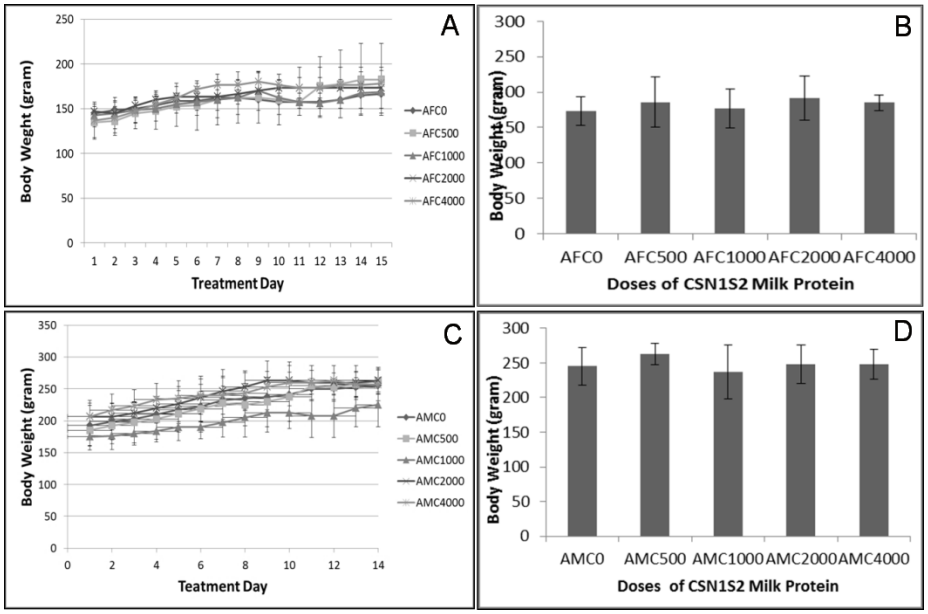

Figure 1 Body weight of rats treated with Caprine CSN1S2 protein from fresh milk in acute toxicity test over 14 days. A and B: body weight of female rats. C and D: body weight of male rats. AFC0 = acute female zero dose of CSN1S2 Protein goat milk; AFC500 = acute female 500 mg/kg BW dose of CSN1S2 Protein goat milk; AFC1000 = AFC1.000 = acute female 1.000 mg/kg BW dose of CSN1S2 Protein goat milk; and AFC2.000 = acute female 2.000 mg/kg BW dose of CSN1S2 Protein goat milk.

3.2 14-day Acute Toxicity Study

In the acute toxicity testing of the male rats there were no differences in the relative weight of the brain, heart, liver, thymus, spleen, kidneys, adrenals, testes, stomach and intestines between treatment groups ($P > 0.05$). Only the lung weight at the dose of 4000 mg/kg BW was significantly higher compared to the control group (Table 1). The histological performance of the lung tissue also showed no abnormal changes in cell morphology (data not shown). The respiratory rate of the animals showed no symptoms of the respiratory distressed syndrome.

Table 1 Relative organ weight of male rats in 14-day acute toxicity test of caprine CSN1S2 protein from fresh milk.

Organs	Doses of Caprine CSN1S2 Protein (mg/kg BW)				
	0	500	1000	2000	4000
Brain	0.70 ± 0.08	0.72 ± 0.04	0.80 ± 0.12	0.70 ± 0.09	0.77 ± 0.09
Heart	0.27 ± 0.02	0.28 ± 0.08	0.34 ± 0.07	0.34 ± 0.07	0.36 ± 0.07
Liver	3.13 ± 1.11	3.04 ± 0.56	2.95 ± 0.59	3.38 ± 0.65	3.41 ± 0.46
Thymus	0.18 ± 0.12	0.22 ± 0.09	0.29 ± 0.13	0.24 ± 0.08	0.22 ± 0.09
Spleen	0.22 ± 0.06	0.23 ± 0.69	0.27 ± 0.07	0.24 ± 0.05	0.27 ± 0.07
Kidney	0.68 ± 0.13	0.55 ± 0.21	0.71 ± 0.08	0.73 ± 0.07	0.79 ± 0.10
Adrenal	0.05 ± 0.08	0.05 ± 0.04	0.10 ± 0.11	0.06 ± 0.05	0.10 ± 0.07
Lung	0.45 ± 0.04	0.52 ± 0.11	0.59 ± 0.10	0.56 ± 0.09	0.64 ± 0.07[a]
Testes	2.28 ± 1.10	1.83 ± 0.77	2.56 ± 0.38	2.73 ± 0.70	2.78 ± 0.50
Stomach	0.73 ± 0.08	0.65 ± 0.09	0.69 ± 0.13	0.77 ± 0.20	0.82 ± 0.16
Intestine	2.88 ± 0.26	2.75 ± 0.24	3.20 ± 0.41	3.18 ± 0.69	3.43 ± 0.61

Note: Data presented as average ± standard deviation; [a] $P < 0.05$ the lung weight in the 4000-dose group was higher compared to the control group.

The acute toxicity testing of the female rats also displayed no differences in relative weight of the brain, heart, liver, thymus, spleen, kidneys, adrenals, ovarian, stomach, lungs and intestines between the treatment groups ($P > 0.05$), as shown in Table 2.

Table 2 Relative organ weight of female rats in 14 day acute toxicity test of caprine CSN1S2 protein from fresh milk.

Organs	Doses of Caprine CSN1S2 Protein (mg/kg BW)				
	0	500	1000	2000	4000
Brain	0.99 ± 0.15	0.89 ± 0.32	0.96 ± 0.09	0.92 ± 0.16	0.99 ± 0.06
Heart	0.36 ± 0.09	0.31 ± 0.06	0.32 ± 0.08	0.34 ± 0.03	0.35 ± 0.04
Liver	3.72 ± 0.47	3.46 ± 0.82	3.59 ± 0.65	3.15 ± 0.27	3.62 ± 0.39
Thymus	0.31 ± 0.07	0.25 ± 0.05	0.29 ± 0.05	0.27 ± 0.05	0.31 ± 0.08
Spleen	0.31 ± 0.12	0.27 ± 0.06	0.30 ± 0.06	0.27 ± 0.01	0.30 ± 0.05
Kidneys	0.73 ± 0.12	0.72 ± 0.16	0.72 ± 0.05	0.77 ± 0.04	0.76 ± 0.13
Adrenals	0.05 ± 0.08	0.06 ± 0.04	0.06 ± 0.02	0.09 ± 0.01	0.10 ±0.04
Lungs	0.69 ± 0.17	0.59 ± 0.13	0.67 ± 0.038	0.69 ± 0.06	0.69 ± 0.11
Ovaries	0.78 ± 0.34	0.69 ± 0.18	0.67 ± 0.142	0.67 ± 0.11	0.77 ± 0.16
Stomach	0.94 ± 0.13	0.94 ± 0.25	0.87 ± 0.136	0.93 ± 0.07	0.87 ± 0.17
Intestines	3.91 ± 0.56	3.69 ± 0.80	4.04 ± 0.76	3.06 ± 0.67	3.86 ± 0.61

Note: Data presented as average ± standard deviation.

3.3 28-day sub-chronic Toxicity Study

In the sub-chronic toxicity study, the male rats showed no differences in the relative weight of the brain, heart, liver, thymus, spleen, kidneys, adrenals, testes, stomach and intestines between treatment groups ($P > 0.05$). However, the obtained liver weight was significantly lower at the dose of 2000 mg/kg BW (2.80 ± 0.60g) compared to the control ($P < 0.05$), as can be seen in Table 3.

Table 3 The relative organ weight for male rats in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Organs	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Brain	0.76 ± 0.08	0.75 ± 0.04	0.87 ± 0.25	0.71 ± 0.03
Heart	0.36 ± 0.05	0.36 ± 0.06	0.36 ± 0.07	0.33 ± 0.05
Liver	3.09 ± 0.39	3.47 ± 0.35	3. 60 ± 0.54	**2.80 ± 0.6**[a]
Thymus	0.19 ± 0.06	0.19 ± 0.05	0.15 ± 0.05	0.18 ± 0.04
Spleen	0.24 ± 0.03	0.25 ± 0.05	0.28 ± 0.09	0.26 ± 0.06
Kidneys	0.67 ± 0.04	0.69 ± 0.06	0.74 ± 0.12	0.65 ± 0.06
Adrenals	0.054 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.01
Lungs	0.62 ± 0.21	0.61 ± 0.16	0.60 ± 0.08	0.51 ± 0.08
Testes	2.52 ± 0.72	2.64 ± 0.79	2.63 ± 0.87	2.86 ± 1.02
Stomach	0.79 ± 0.07	0.80 ± 0.03	1.25 ± 0.61	0.79 ± 0.10
Pancreas	0.34 ± 0.11	0.35 ± 0.08	0.29 ± 0.13	0.27 ± 0.07

Note: Data presented as average ± standard deviation; [a] $P < 0.05$ the liver weight for the 2000-dose group was lower than that for the other groups.

Similar to the relative weight of some organs of the male rats, the sub-chronic toxicity test showed that in the female rats there were no statistical differences in the relative weight of the brain, heart, liver, thymus, spleen, kidneys, lungs, adrenals, ovaries, gastric tracts and pancreas between groups ($P > 0.05$), as can be seen in Table 4.

Table 4 The relative organ weight for female rats in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Organs	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Brain	0.92 ± 0.12	0.94 ± 0.07	0.915 ± 0.17	0.90 ± 0.04
Heart	0.33 ± 0.07	0.34 ± 0.04	0.32 ± 0.03	0.38 ± 0.046
Liver	3.10 ± 0.47	3.21 ± 0.69	3.63 ± 0.52	3.43 ± 0.25
Thymus	0.25 ± 0.46	0.26 ± 0.07	0.27 ± 0.08	0.20 ± 0.07
Spleen	0.26 ± 0.06	0.26 ± 0.04	0.27 ± 0.04	0.28 ± 0.09
Kidneys	0.70 ± 0.87	0.73 ± 0.15	0.74 ± 0.13	0.65 ± 0.18
Adrenals	0.10 ± 0.10	0.11 ± 0.13	0.11 ± 0.13	0.13 ± 0.12
Lungs	0.71 ± 0.19	0.74 ± 0.24	0.61 ± 0.13	0.54 ± 0.24
Ovaries	0.79 ± 0.26	0.81 ± 0.23	0.88 ± 0.38	0.72 ± 0.23
Stomach	0.95 ± 0.15	0.97 ± 0.89	0.91 ± 0.07	0.88 ± 0.16
Pancreas	0.42 ± 0.12	0.45 ± 0.16	0.42 ± 0.17	0.37 ± 0.14

Note: Data presented on average ± standard deviation.

All hematological data (Tables 5 and 6) and clinical chemistry data (Tables 7 and 8) show no significant differences between groups of both sexes ($P > 0.05$). The caprine casein protein from fresh milk was not potentially toxic after the 28 day sub-chronic toxicity test and there were no treatment-related changes in hematological and clinical test parameters.

Table 5 Hematological characterization of male rat blood in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Hematological Parameters	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Hemoglobin (g/dl)	15.20 ± 0.95	17.47 ± 6.52	15.266 ± 4.00	14.00 ± 1.97
WBC (x 10^3/µL)	29.00 ± 0.11	43.467 ± 6.99	66.00 ± 4.10	37.93 ± 6.18
Hematocrit (%)	28.50 ± 0.74	27.13 ± 15.38	28.33 ± 17.79	25.67 ± 18.70
Trombocyte (x 10^3/µL)	1160000 ± 150000	1358000 ± 414231.8	1293333.33 ± 481307.9	1067333.33 ± 330165.6
RBC (x 10^6/µL)	49.70 ± 1.12	43.02 ± 36.17	45.20 ± 3.37	46.10 ± 3.55
MCV (fL)	60.67 ± 3.60	62.67 ± 10.79	66.33 ± 12.86	61.67 ± 12.42
MCH (pg)	81.03 ± 34.86	80.93 ±103.20	100.13 ± 137.67	79.63 ± 101.47
MCHC (g/dL)	117.10 ± 45.73	114.57 ±132.87	129.33 ± 164.08	112.20 ± 127.24

Note: WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 6 Hematological characterization of female rat blood in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Hematological parameters	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Hemoglobin (g/dl)	14.33 ± 2.02	14.33 ± 0.81	13.33 ± 1.45	13.53 ± 1.21
WBC (x 10^3/µL)	28.00 ± 14.00	23.33 ± 3.05	28.00 ± 2.00	26.00 ± 5.15
Hematocrit (%)	38.20 ± 4.161	36.53 ± 0.611	34.80 ± 4.39	35.33 ± 2.48
Trombocyte (x 10^3/µL)	1038000 ± 196611.3	818000 ± 172835.2	906000 ± 213100.0	1070666.67 ± 158396
RBC (x 10^6/µL)	26.80 ± 0.45	26.43 ± 34.62	24.13 ± 30.90	26.42 ± 35.84
MCV (fLl)	58.00 ± 2.65	56.00 ± 1.00	56.00 ± 2.645	58.00 ± 2.00
MCH (pg)	21.70 ± 1.65	22.00 ± 0.69	21.50 ± 0.70	22.37 ± 1.44
MCHC (g/dL)	37.30 ± 1.32	39.23 ± 1.50	38.30 ± 1.22	38.47 ± 1.72

Note: WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 7 Clinical characteristics of male rat blood serum in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Level	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Glucose (mg/dL)	78.00 ± 32.05	49.00 ± 21.93	50.67 ± 20.50	51.00 ± 11.14
TCHO (mg/dL)	73.33 ± 16.01	62.67 ± 17.62	83.33 ± 20.60	73.33 ± 9.24
Triglyceride (mg/dL)	92.33 ± 22.59	76.67 ± 45.28	90.00 ± 32.19	75 ± 25.63
BUN (mg/dL)	24.43 ± 2.48	20.93 ± 3.86	27.83 ± 10.22	18.27 ± 4.37
Creatinin (mg/dL)	0.70 ± 0.06	0.75 ± 0.25	0.64 ± 0.13	0.64 ± 0.01
Uric acid (mg/dL)	3.77 ± 1.10	3.27 ± 0.99	15.87 ± 23.50	2.40 ± 0.43
ALP (IU/dL)	288.00 ± 52.51	316.33 ± 170.12	253.00 ± 143.81	260.00 ± 38.74
AST (IU/dL)	179.33 ± 51.08	157.33 ± 58.77	183.33 ± 76.27	168.67 ± 87.68
ALT (IU/dL)	56.00 ± 20.81	61.33 ± 35.44	67.67 ± 48.95	90.33 ± 52.50
Total Protein (g/dL)	10.10 ± 0.49	9.02 ± 1.99	8.51 ± 0.66	8.78 ± 0.29
Bilirubin (mg/dL)	1.36 ± 0.00	1.46 ± 0.25	1.06 ± 0.13	1.36 ± 0.45

Note: TCHO: total cholesterol; BUN: blood urea nitrogen; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Table 8 Clinical characteristics of female rat blood serum in 28-day sub-chronic toxicity test of caprine CSN1S2 protein from fresh milk.

Level	Doses of Caprine CSN1S2 Protein (mg/kg BW)			
	0	500	1000	2000
Glucose (mg/dL)	59.67 ± 29.36	65.67 ± 12.50	64.00 ± 19.16	77.67 ± 9.81
TCHO (mg/dL)	71.67 ± 2.31	70.67 ± 18.58	80.33 ± 20.01	70.33 ± 4.51
Triglyceride(mg/dL)	78.67± 35.92	87.33 ± 35.47	87.67 ± 31.08	139.67 ± 75.05
BUN (mg/dL)	26.93 ± 6.48	30.47 ± 8.19	26.57 ± 4.31	25.83 ± 0.12
Creatinin (mg/dL)	1.05 ± 0.23	1.09 ± 0.69	0.96 ± 0.50	1.01 ± 0.43
Uric acid (mg/dL)	3.97 ± 1.15	3.00 ± 1.10	4.00 ± 2.55	3.50 ± 1.48
ALP (IU/dL)	235.67 ± 130.20	145.33 ± 40.82	209.33 ± 142.53	218.00 ± 136.47
AST (IU/dL)	178.33 ± 68.122	213.33 ± 45.08	199.67 ± 5.77	154.333 ± 43.29
ALT (IU/dL)	77.67 ± 35.30	61.67 ± 29.36	64.00 ± 30.05	71.33 ± 15.57
Total Protein (g/dL)	8.92 ± 0.89	9.76 ± 1.75	9.65 ± 1.75	10.36 ± 1.39
Bilirubin (mg/dL)	1.26 ± 0.37	1.06 ± 0.14	0.96 ± 0.16	1.22 ± 0.29

Note: TCHO: total cholesterol; BUN: blood urea nitrogen; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

4 Discussion

Our previous study showed that caprine CSN1S2 protein is able to stimulate ileum toxicity up to 2000 mg/kg body weight dosage. At a dose of 2 mg/kg body weight, CSN1S2 from goat milk is able to decrease the severity scoring, TNF-a, and RAGE expression in a complete Freund's adjuvant induced rheumatoid arthritis model in rats. Besides, this compound also acts as anti-osteoporotic agent [6,12,15]. In this study, acute and chronic toxicity testing of male and female rats treated with caprine CSN1S2 protein from fresh milk was conducted. In the 14-day acute toxicity test, the daily food and water consumption was normal. Body weight and daily body weight (data not shown) of the rats in all groups increased slightly. This result indicates no abnormal effects from treatment with caprine CSN1S2 protein from fresh milk in both rat sexes. Recently, a study using bovine CSN1S1 protein reported that body weight, daily body weight gain, food and water consumption were unaffected by the treatment with a single oral dose of 2000 mg/kg of bovine alpha-S1 casein hydrolysate [13].

The most sensitive toxicity study indicators are organ weight – in the absence of morphological changes – and age, sex, animal strain, and experimental conditions [16]. In the acute toxicity tests, interval doses of caprine CSN1S2 protein from fresh milk were used. Our results indicate that the male rats' lung weight gain at a high dose (4000 mg/kg BW) may be seen as an early lung/respiratory response caused by the caprine casein protein of fresh milk consumption. Meanwhile, the respiratory rate data show that none of the animal groups displayed any symptoms related to the respiratory distress syndrome.

Caprine casein protein from fresh milk may cause fluid to accumulate in the rats' lungs and oxygen levels in the blood to be normal, respectively.

The highest organ weight gain in the male rats in the 28-day sub-chronic toxicity test was not abnormal, although the liver weight was slightly elevated at doses of 500-1000 mg/kg BW and declined at a dose of 2000 mg/kg BW. These slight changes in the organ weight of the animals are probably not to be considered treatment effects because these values are within the normal weight ranges [17,18]. Another study predicted elevation of the liver weight in less than 7 days duration because of the induction of potent hepatic enzyme compounds [19]. Meanwhile, the female rat organs displayed no change in the relative weights of the brain, heart, liver, thymus, spleen, kidneys, lungs, adrenals, ovaries, gastric tracts and pancreas between groups. According to this study, it can be recommended that milk casein protein toxicity studies based on the weight of organs may focus on detecting test article-related effects, such as steroid and non-steroid hormone levels, hepatic enzyme compounds, or other environmental factors.

The hematopoietic systems are very sensitive to toxic compounds and are subject to change due to the intake of toxic compounds from plants or food. Changes in the hematopoietic system have a high predictive value for toxicity in humans when data are translated from animal studies. Bone marrow activity status and intravascular effects can be monitored through changes in hematological parameters [18,20,21]. In this study, several hematological parameters showed no significant changes between the treatment groups and the control group. This indicates that CSN1S2 is not toxic to the hematopoietic system.

Analysis of blood chemistry was performed to assess toxicity to the functions of the pancreas (glucose), kidneys (BUN, creatinine) and liver (SGOT, SGPT, ALP, total protein, albumin, and bilirubin) [22-24]. This study found no significant changes in these clinical chemistry parameters in any of the male and female rats treated with caprine CSN1S2 protein from fresh milk. This indicates that the oral administration of sub-chronic caprine CSN1S2 protein from fresh milk does not interfere with the function of the pancreas, kidneys or liver. In addition, providing caprine CSN1S2 protein from fresh milk also does not affect cholesterol and triglyceride levels in the lipid metabolism. Our studies confirmed that a safe dose of caprine CSN1S2 protein from fresh milk from local goats is up to 2000 mg/kg BW. Recently, milk casein protein toxicity effect studies have shown that a daily dose of 1000-2000 mg/kg of body weight for 4 weeks is well tolerated in rats. No systemic evidence was found for mortality or alterations of the hematological or clinical pathology, and there were no changes in body weight or body weight gain after daily consumption of

casein for 4 weeks [17]. Our investigation of the rats' small intestine microstructures and mineral profiles showed no abnormalities for a dose of 2000 mg/kg BW, i.e. the maximum recommended daily dose of CSN1S2 protein isolated from casein protein goat milk [12].

5 Conclusion

From this study, it can be concluded that caprine CSN1S2 protein from fresh milk of local goats does not cause symptoms or signs of toxicity and can be recommended as safe for food consumption. Caprine CSN1S2 protein from fresh milk also does not trigger lethal effects or changes in hematological and blood chemistry parameters.

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