# Acute and repeated dose (4 weeks) oral toxicity studies of two antihypertensive peptides, RYLGY and AYFYPEL, that correspond to fragments (90–94) and (143–149) from $a_{s1}$-casein

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## Abstract

The Lowpept® is a powdered casein hydrolysate containing the antihypertensive peptides RYLGY and AYFYPEL, two sequences that correspond to as1-casein f (90–94) (RYLGY)1 and as1-casein f (143–149) (AYFYPEL)1. To support the safety, Lowpept® has been examined in an acute and in a 4-week repeated dose oral toxicity studies in rats. Powdered casein hydrolysate administered in a single oral gavage dose of 2000 mg/kg resulted in no adverse events or mortality. Also, casein hydrolysate administered as a daily dose of 1000 mg/kg for 4 weeks by gavage resulted in no adverse events or mortality. No evidence or treatment-related toxicity was detected during both studies. Data analysis of body weight gain, food consumption, clinical observations, blood biochemical, haematology, organ weight ratios and histopathological findings did not show significant differences between control and treated groups. It is concluded that the casein hydrolysate containing the peptides RYLGY and AYFYPEL orally administered to rats was safe and that not treatment-related toxicity was detected even at the highest doses investigated in both acute (2000 mg/kg of body weight) and repeated dose (4 weeks) oral (1000 mg/kg of body weight) toxicity studies.

*Keywords:*
Casein hydrolysate
RYLGY and AYFYPEL antihypertensive peptides
Safety evaluation

## 1. Introduction

Milk proteins exert a wide range of nutritional and biological properties. Recently, increasing attention is being focused on physiologically active peptides that could be potential ingredients of health-promoting foods. Hypertension has become a serious health problem, especially in developing countries, and it has been considered a risk factor for the developing cardiovascular diseases. Due to the important role of diet in the prevention and treatment of hypertension, particular interest has been given in the production of foods with antihypertensive activity. Antihypertensive peptides are mainly effective in the treatment of hypertension by inhibiting angiotensin I-converting enzyme (ACE) which plays a key role for regulation of blood pressure through the rennin-angiotensin system (Ondetti et al., 1977). The consumption of food products containing antihypertensive peptides produces a significantly reduction in blood pressure (Jauhiainen and Korpela, 2007).

The enzymatic hydrolysis of milk proteins using microbial and digestive enzymes and fermentation with different microorganisms are the most common strategies to produce antihypertensive peptides. A long number of ACE-inhibitory and antihypertensive peptides derived from milk proteins have been reported so far (FitzGerald and Murray, 2006; López-Fandiño et al., 2006; Quirós et al., 2007; Hernández-Ledesma et al., 2008; Philanto et al., 2010). Among of them, the most potent antihypertensive peptides were generated from caseinate or casein fractions, probably related to the abundance of proline residues in the casein fractions (Otte et al., 2007). The first fermented milk with antihypertensive activity was produced with a combination of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* and contained two ACE-inhibitory tripeptides, isoleucine–proline–proline (IPP) and valine–proline–proline (VPP) (Nakamura et al., 1995). These two tripeptides have also been produced by milk fermentation with *Lactobacillus helveticus* LBK-16H (Seppo et al., 2003) and casein hydrolysis with a protease from *Aspergillus oryzae* (Mizuno et al., 2004). These tripeptides exhibited potent ACE-inhibitory activity and proven antihypertensive effects in both animals and humans (Sipola et al., 2002; Hata et al., 1996). Xu et al. (2008) in a meta-analysis of randomized controlled trials showed that IPP and VPP had hypotensive effects in prehypertensive and hypertensive subjects. These peptides are probably the most extensively studied bioactive peptides from caseins and have been included in commercialized functional foods (Korhonen, 2009).

Table 1. Haematological and clinical biochemistry parameters.

| Haematological parameters |
|---|
| Red blood cell count (RBC) Haemoglobin Haematocrit |
| Mean corpuscular volume (MCV) Mean corpuscular haemoglobin (MCH) |
| Mean corpuscular haemoglobin concentration (MCHC) |
| Nucleated red blood cell count (RDW) |
| White blood cell count (WBC) Band neutrophils count Neutrophils count |
| Eosinophils count Lymphocytes count Monocytes count |
| Basophils count Platelet count |
| Mean platelet volume (MPV) Prothrombin time (28 daysThromboplastin partial time (28 days) Fibrinogen (28 days) |
| *Clinical biochemistry parameters* |
| Glucose Urea Creatinine |
| Total protein |
| Total bilirubin Calcium |
| Sodium Potassium |
| Aspartate aminotransferase (ASAT) Alanine aminotranferase (ALAT) Alkaline phosphatase |
| Triglyceride Cholesterol |
| High density lipoproteins (HDL) Low density lipoproteins (LDL) Albumine (28 days) |
| Lipoprotein A (28 days) |

Because the cost of production of peptides by enzymatic hydrolysis is less expensive and more versatile than fermentation, in our laboratory we have used digestive enzymes as pepsin to produce a casein hydrolysate with ACE-inhibitory activity and antihypertensive properties (Miguel et al., 2009). Novel casein-derived sequences with antihypertensive and antioxidant activity have been identified in a peptic casein hydrolysate using RP–HPLC– MS/MS analysis (Contreras et al., 2009). Among the 44 identified peptides, two sequences, that corresponded to fragments 90–94 (RYLGY) and 143–149 (AYFYPEL) of $\alpha_{s1}$-CN showed angiotensin-converting enzyme-inhibitory activity expressed as $IC_{50}$ values as low as 0.71 1M and 6.58 1M, respectively. These two peptides also exerted potent antihypertensive activity when they were orally administered to spontaneously hypertensive rats (strain SHR) at a dose of 5 mg/kg of body weight and the antihypertensive effect was similar to that found for VPP (Contreras et al., 2009).

Although there are numerous reports about the production of potential antihypertensive peptides from food proteins, there are few studies about the possible adverse effects that might be exerted by the peptides or their metabolites. Toxicology studies are necessary to asses the safety of a functional food previous commercialization. Mizuno et al. (2005) assessed the toxicological potential of a powdered casein hydrolysate containing two tripeptides (VPP and IPP) when administered once daily for 13-weeks to rats. The results of these studies suggested that there was no evidence of target organ toxicity associated with the administration of the tripeptides at a dose of 1000 mg/kg body weight per day. The aim of this study was to evaluate the potential acute and repeated dose (4 weeks) oral toxicity of hydrolyzed casein containing the new sequences RYLGY and AYFYPEL with antihypertensive activity.

## 2. Materials and methods

### *2.1. Casein hydrolysate*

The test substance (Lowpept®) is a powdered casein hydrolysate. It was prepared by casein hydrolysis with food-grade pepsin (Biocatalysts, Cardiff, UK) as described in a previous paper (Contreras et al., 2009) with slight modifications (Recio et al., 2006). The reaction was terminated by heating at 80–85 °C for 15 min and the pH was

adjusted to pH 4,0 with addition of 1 M KOH. The resulting hydrolyzed casein was subsequently spray-dried to produce the Lowpept® powder. This casein hydrolysate contains two antihypertensive peptides with sequences that correspond to *as*1-casein *f* (90–94) (RYLGY) and *as*1-casein *f* (143–149) (AYFYPEL). The protein concentration of powdered hydrolysate was determined by Kjeldahl method according to the standard method of International Dairy Federation (1993). Moisture was determined by drying for 3 h in an oven at 100–105 °C following the standard method of International Dairy Federation (1964). Ash content was determined by ignition of solid materials at 550 °C in an electric muffle furnace (Selecta, Barcelona, Spain). Mineral composition (Na, K, Ca, and Mg) was determined by atomic absorption spectroscopy following the method described by De la Fuente et al. (1997). An atomic absorption spectrometer (mod 5100 PC; Per- kin–Elmer Norwalk, CT) with an air and acetylene-oxidizing flame was used. Sodium and K were determined by atomic emission at wavelengths of 589 and 766.5 nm, respectively. Calcium and Mg were measured by atomic absorption with a multielement hollow cathode lamp. Microwave digestion was carried out in a microwave oven (mod Ethos 1; Milestone Shelton, CT, USA).

### *2.2. Determination of ACE-inhibitory activity*

ACE-inhibitory activity was measured by fluorescence using the method of Sentandreu and Toldrá (2006), with some modifications (Quirós et al., 2009). The ACE (peptidyl-dipeptidase A, EC 3.4.15.1) (Sigma Chemical, St. Louis, MO, USA) was prepared at 50% glycerol:water (1 U/ml) and conserved at —20 °C. ACE working solution was diluted with 0.15 M tris buffer (pH 8.3) containing 0.1 1M $ZnCl_2$ with 0.04 U/ml of enzyme in the final reaction solution. A total of 40 1l of distilled water or this working solution were added to each microtiter-plate well, then adjusted to 80 1l by adding distilled water to blank (B), control (C) or samples (S). The enzyme reaction was started by adding 160 1l of 0.45 mM o-Abz-Gly-p-Phe($NO_2$)-Pro-OH (Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at 37 °C. The fluorescence generated was measured at 30 min using a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). Ninety-six-well microplates (Porvair, Leatherhead, UK) were used. Excitation and emission wavelengths were 350 and 420 nm, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech). Activity was tested in triplicate. Inhibitory activity was expressed as the protein concentration required inhibiting the original ACE activity by 50% ($IC_{50}$).

### 2.3. *Quantification of peptides RYLGY and AYFYPEL in the casein hydrolysate*

The amount of peptides RYLGY and AYFYPEL in the powdered casein hydrolysate was estimated by reverse phase high performance liquid chromatography (RP–HPLC) coupled to tandem mass spectrometry (MS/MS). To prepare standard solutions, the peptides RYLGY and AYFYPEL synthesized by Fmoc solid-phase synthesis were purchased from GenScript Corp. (Piscataway, NJ, USA). Stock solutions of the synthetic peptides were prepared at 1 mg/ml in Milli-Q water, and then diluted to prepare six calibration points from 1 to 40 μg/ml. The spray-dried casein hydrolysate was dissolved in Milli-Q water at 5 mg/ml.

RP–HPLC separations of the hydrolysates were performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source, as previously described (Contreras et al., 2010). The variable-wavelength detector was set at 214 nm. A $C_{18}$-guard column (Nova-Pak® 20 mm × 3.9 × 4 μm of particle size; Waters Corp., Milford, MA, USA) was used to protect the analytical column (HiPore® RP318 $C_{18}$ column 250 × 4.6 mm and 5 lm of particle size; Bio-Rad, Richmond, CA, USA). The samples were eluted at a flow rate of 0.8 ml/min with a linear gradient from 0 to 45% of solvent B [acetonitrile and trifluoroacetic acid (TFA), 1000:0.270, v/v] in solvent A (Milli-Q water and TFA, 1000:0.370, v/v) in 60 min. The injection volume was 50 ml and a duplicate of injection was made for each point of the standard curve and the samples. The flow from HPLC was divided approximately 1:3 previous to ionization source. For HPLC–MS, spectra were recorded over the mass-to-charge (*m/z*) range of 100–1500. About 15 spectra were averaged in the MS analyses and about 5 spectra in the tandem MS analyses. Using Data Analysis (version 3.0; Bruker Daltoniks), the *m/z* spectral data were processed and transformed to spectra representing mass values.

Plots were made of the HPLC–MS peak area of the molecular ions with *m/z* value of 671.2 and 902.2 corresponding to RYLGY and AYFYPEL, and their sodium and potassium adducts (*m/z* 693.2, 709.2 and 924.2, 940.2, respectively) vs. peptide concentration.

Table 2. Composition and content in active peptides of the food-grade casein hydrolysate Lowpept®.

| | Powdered casein hydrolysate (Lowpept®) |
|---|---|
| Total protein (%) | 66.5 |
| Moisture (%) | 4.8 |
| Ash (%) | 13.1 |
| Calcium (mg/g) | 26.19 |
| Magnesium (mg/g) | 1.18 |
| Sodium (mg/g) | 4.34 |
| Potassium (mg/g) | 24.95 |
| RYLGY (mg/g) | 1.52 |
| AYFYPEL (mg/g) | 2.46 |

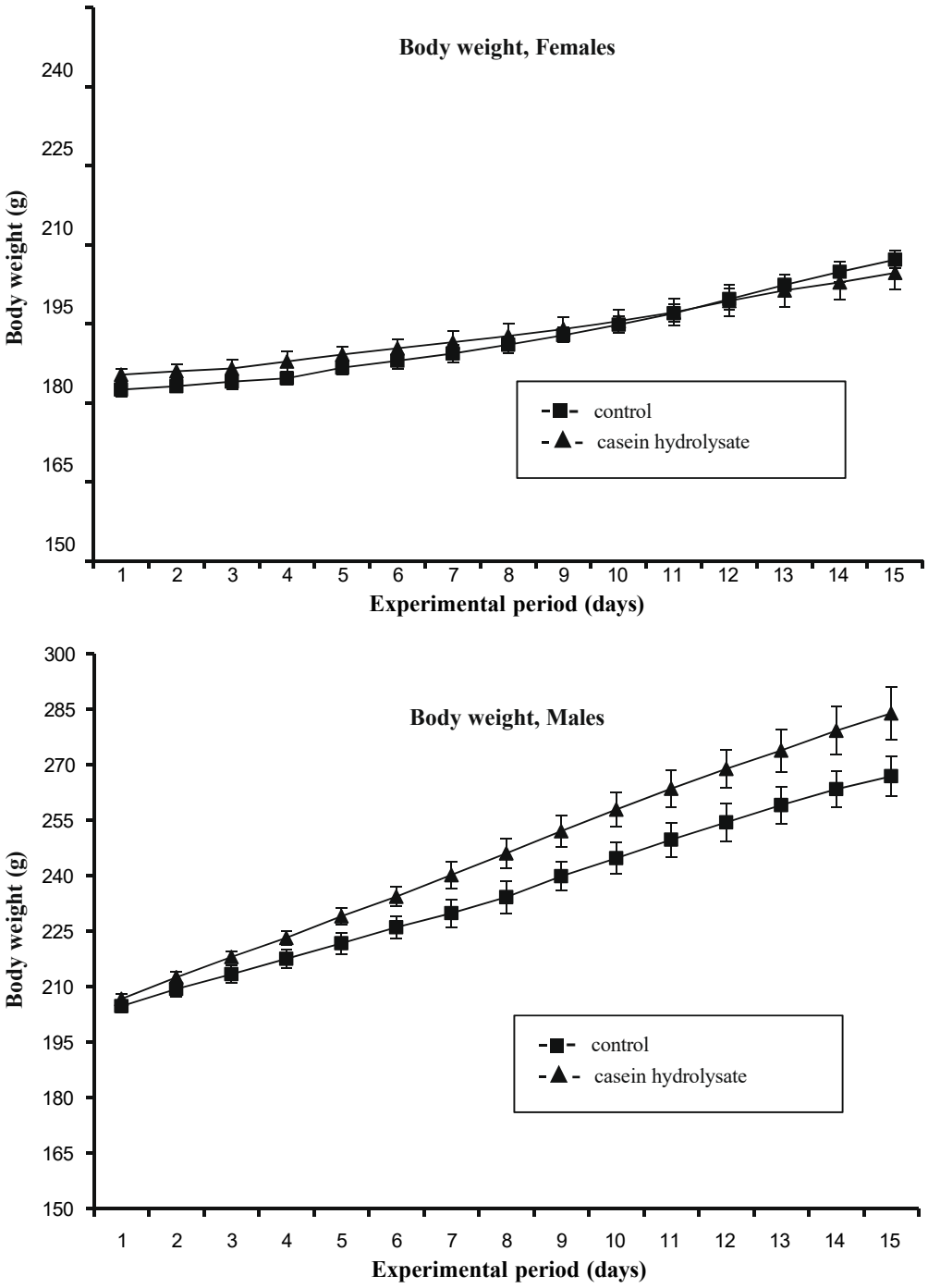


Fig. 1. Body weight of rats during the 2-week observation period, following a single oral dose of Lowpept® at 2000 mg/kg of body weight. Mean values, *n* = 6 animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.

### *2.4. Acute and repeated dose (4-weeks) oral toxicity studies*

Wistar male and female rats (Charles River Inc., Marget, Kent, UK) were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50% ± 10% relative humidity) with a 12 h light–dark cycle (light from 08.00 to 20.00 h). Food (A03 rodent diet, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) and water were available ad libitum. The rats were 56-days old at the initiation of treatment. Acute (limit test) and repeated dose (4 weeks) studies were conducted in accordance with the European Union guidelines (EC Council Regulation No. 440, 2008a,b). Both studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University.

In the acute (limit test) study, 24 rats (12 males, 12 females) were distributed into two groups of 6 males and 6 females each. After an overnight fast each rat received distillate water orally (control group or Group 1), or a single oral dose of 2000 mg/kg of Lowpept® product dissolved in distilled water (treated group or Group 2). Doses of the test and control articles were administered by gavage at a volume of 8 ml/kg body weight based on the individual animal body weights obtained on the day dosing. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). At the end of a 14 days observation period, the rats were weighed, euthanized by CO2 inhalation, exsanguinated, and necropsied.

The repeated dose (4 weeks) (limit test) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group or Group 3; treated group or Group 4; satellite control group or Group 5; and satellite treated group or Group 6). Rats received a daily dose of either distilled water (Groups 3 and 5) or 1000 mg/kg body weight of the Lowpept® product dissolved in distilled water (Groups 4 and 6) orally once a day over 4 weeks. Doses of the test and control articles were administered by gavage at a volume of 4 ml/ kg body weight based on the individual animal body weights obtained on the day dosing. Animals were dosed at approximately the same time each day (approximately 4–6 h into light cycle). Food but not water was withheld from 4 h before until 2 h after control and test article administration. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). All rats of the Groups 3 and 4 were deprived of food for 18 h, weighed, euthanized by CO2 inhalation, exsanguinated, and necropsied on Day 29. All animals of the satellite groups (Groups 5 and 6) were kept a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects. All rats of the Groups 5 and 6 were deprived of food for 18 h, weighed, euthanized by CO2 inhalation, exsanguinated, and necropsied on Day 42.

#### *2.4.1. Observations*

All animals were observed twice daily for general appearance, behaviour, sings of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, evaluated for respiration rate and palpated for masses. Behavioural parameters checked were abnormal movements (tremor, convulsion, muscular contractions), reactions to handling and behaviour in open field (excitability, responsiveness to touch and to sharp noise), changes in ordinary behaviour (changes in grooming, head shaking, gyration), abnormal behaviour (autophagia, backward motion) and aggression. Body weight, body weight gain and food and water consumption were measured daily and at the end of the observation periods the rats were examined by necropsy, and the weights of the organs recorded.

#### *2.4.2. Clinical test parameters*

Blood samples for haematology and clinical chemistry evaluation were collected from the retro-orbital plexus from animals under light anesthesia induced by CO2 inhalation after 14 days observation period in the acute oral study and after 4 weeks of treatment and 14 days of recovery for the repeated dose 4 weeks safety study. EDTA was used as an anticoagulant for haematology samples and sodium citrate was used as an anticoagulant for clinical chemistry. Food was withheld for approximately 18 h before blood collection, and samples were collected early in the working day to reduce biological variation; water was provided ad libitum. Clinical pathology parameters (haematological and clinical biochemistry) were evaluated (Table 1). Most haematology variables were measured with a Coulter/ CELL-DYN 3500 whole blood automated analyzer (Abbott, Chicago, IL). Blood cell smears were observed with an Olympus Microscopy BX41 (Olympus, Tokyo, Japan). Clinical chemistry parameters were evaluated with a spectrophotometer Konelab PRIME 30 (Thermofisher Scientific Inc. Waltham, MA, USA) and special biochemistry parameters with a clinical chemistry analyzer AU640 (Olympus, Tokyo, Japan). Coagulation parameters were analyzed with a coagulation analyzer Coatron M1 (Teco Medical Instruments, GMBH, Neufahrn, Germany).

Table 3. Haematological parameters in rats after the 2-week observation period following a single oral dose of Lowpept® at 2000 mg/kg of body weight.

| Parameters[a] | Group 1 (Control) | | Group 2 (Lowpept® 2000 mg/kg) | |
|---|---|---|---|---|
| | Female | Male | Female | Male |
| *Acute oral dose* | | | | |
| RBC ($\times 10^6$/1l) | 8.10 ± 0.19 | 8.49 ± 0.04 | 8.46 ± 0.12 | 8.10 ± 0.19 |
| Haemoglobin (g/dl) | 14.75 ± 0.38 | 14.80 ± 0.23 | 15.07 ± 0.14 | 15.78 ± 0.44 |
| Haematocrit (%) | 47.33 ± 0.68 | 48.40 ± 0.39 | 48.12 ± 0.56 | 47.37 ± 0.59 |
| MCV (fl) | 58.50 ± 0.62 | 57.00 ± 0.36 | 56.83 ± 0.87 | 58.50 ± 1.06 |
| MCH (pg) | 18.22 ± 0.26 | 17.43 ± 0.22 | 17.70 ± 0.41 | 17.95 ± 0.15 |
| MCHC (g/dl) | 31.13 ± 0.43 | 32.25 ± 0.26 | 31.03 ± 0.60 | 32.58 ± 0.24 |
| RDW (%) | 15.33 ± 0.12 | 15.92 ± 0.11 | 15.05 ± 0.28 | 15.67 ± 0.35 |
| WBC ($\times 10^3$/1l) | 8.45 ± 0.57 | 9.63 ± 0.76 | 8.68 ± 0.71 | 11.63 ± 0.75 |
| Banded neutrophils ($\times 10^3$/1l) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Neutrophils ($\times 10^3$/1l) | 1.59 ± 0.17 | 1.67 ± 0.18 | 1.31 ± 0.10 | 1.37 ± 0.15 |
| Eosinophils ($\times 10^3$/1l) | 0.03 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.03 | 0.02 ± 0.02 |
| Lymphocytes ($\times 10^3$/1l) | 6.52 ± 0.45 | 8.95 ± 0.40 | 7.05 ± 0.64 | 9.59 ± 0.57 |
| Monocytes ($\times 10^3$/1l) | 0.32 ± 0.11 | 0.42 ± 0.09 | 0.27 ± 0.07 | 0.32 ± 0.06 |
| Basophils ($\times 10^3$/1l) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Platelets ($\times 10^3$/1l) | 684.00 ± 16.02 | 709.83 ± 9.80 | 722.67 ± 26.69 | 731.50 ± 37.94 |
| MPV (fl) | 6.60 ± 0.07 | 6.78 ± 0.11 | 6.05 ± 0.17 | 6.87 ± 0.12 |

Differences between the treated group and the control group were not significant.
[a] Data are expressed as mean ± SEM ($n$ = 6 animals/sex group).

Table 4. Clinical chemistry parameters in rats after the 2-week observation period following a single oral dose of Lowpept® at 2000 mg/kg of body weight.

| Parameters[a] | Group 1 (Control) | | Group 2 (Lowpept® 2000 mg/kg) | |
|---|---|---|---|---|
| | Female | Male | Female | Male |
| *Acute oral dose* | | | | |
| Glucose (mg/dl) | 111.50 ± 3.01 | 118.83 ± 3.82 | 122.67 ± 4.86 | 122.17 ± 5.84 |
| Urea nitrogen (mg/dl) | 49.17 ± 3.61 | 43.00 ± 2.32 | 49.00 ± 1.51 | 43.33 ± 1.73 |
| Creatinine (mg/dl) | 0.57 ± 0.03 | 0.55 ± 0.02 | 0.63 ± 0.03 | 0.53 ± 0.02 |
| Total protein (g/dl) | 7.52 ± 0.12 | 6.75 ± 0.04 | 7.50 ± 0.24 | 6.97 ± 0.23 |
| Total bilirubin (mg/dl) | 0.15 ± 0.02 | 0.15 ± 0.02 | 0.17 ± 0.02 | 0.18 ± 0.02 |
| Calcium (mg/dl) | 11.26 ± 0.24 | 11.39 ± 0.21 | 11.08 ± 0.14 | 11.91 ± 0.21 |
| Sodium (mEq/l) | 147.17 ± 2.37 | 145.17 ± 1.28 | 146.83 ± 0.95 | 150.00 ± 2.78 |
| Potassium (mEq/l) | 6.08 ± 0.46 | 6.42 ± 0.60 | 5.73 ± 0.26 | 6.58 ± 0.35 |
| ASAT (u/l) | 149.83 ± 6.67 | 146.33 ± 6.92 | 152.50 ± 7.44 | 165.33 ± 7.23 |
| ALAT (u/l) | 64.33 ± 3.68 | 69.50 ± 4.68 | 63.33 ± 2.78 | 73.33 ± 3.19 |
| Alkaline phosphatase (u/l) | 296.33 ± 25.33 | 830.83 ± 29.72 | 264.67 ± 21.33 | 820.83 ± 43.11 |
| Triglyceride (mg/dl) | 114.83 ± 11.48 | 114.33 ± 4.51 | 103.33 ± 12.45 | 130.83 ± 7.67 |
| Cholesterol (mg/dl) | 65.17 ± 2.47 | 57.00 ± 2.97 | 68.33 ± 2.09 | 63.33 ± 3.79 |
| HDL (mg/dl) | 48.64 ± 1.76 | 39.82 ± 1.60 | 46.61 ± 1.96 | 42.90 ± 2.52 |
| LDL (mg/dl) | 8.06 ± 0.78 | 8.18 ± 0.80 | 7.31 ± 0.43 | 8.54 ± 0.60 |

Differences between the treated group and the control group were not significant.
[a] Data are expressed as mean ± SEM ($n$ = 6 animals/sex group).

### *2.4.3. Anatomical pathology*

All rats were euthanized by $CO_2$ inhalation and necropsied. The necropsy included a macroscopic examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal, and pelvic cavities and viscera. Descriptions of all macroscopic abnormalities were recorded. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate-buffered 4% formaldehyde solution: adrenal glands, brain, heart, ileum, jejunum, caecum, colon, duodenum, rectum, stomach, oesophagus, trachea, kidneys, liver, lungs, pancreas, spleen, skin, testicles with epididymes, ovaries with oviducts, bone marrow, thymus, thyroid and parathyroid glands, seminal vesicles, urinary bladder and uterus. The organ:body weight ratios were calculated. All organ and tissue samples for histopathological examination were processed, embedded in paraffin, cut at an approximate thickness of 2 to 4 lm, and stained with hematoxylin and eosin. Slides of all organs and tissues listed above were collected from all animals of the control and treated groups.

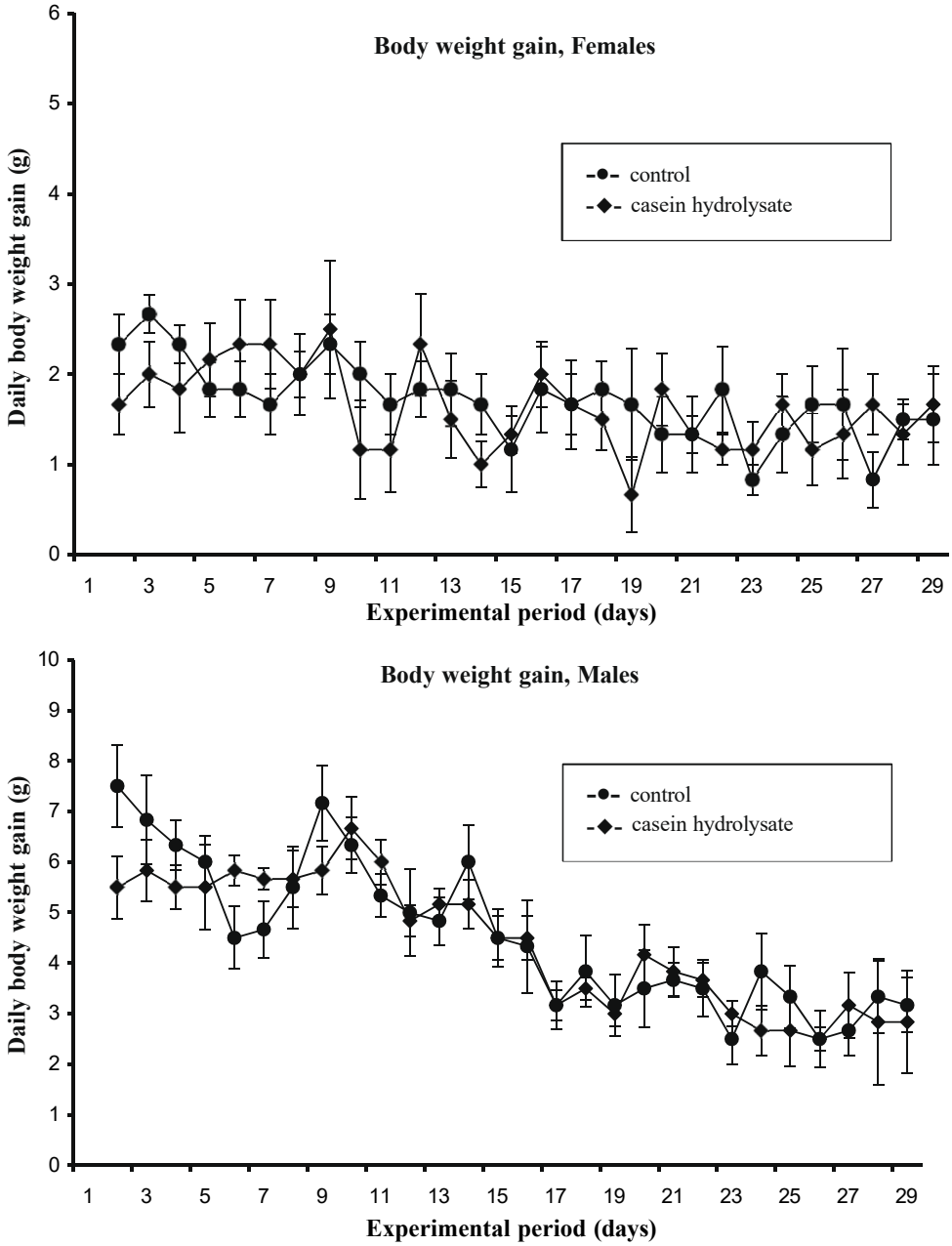


Fig. 2. Daily body weight gain of rats exposed to repeated (4 weeks) oral doses of Lowpept® at 1000 mg/kg of body weight. Mean values, $n = 6$ animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.

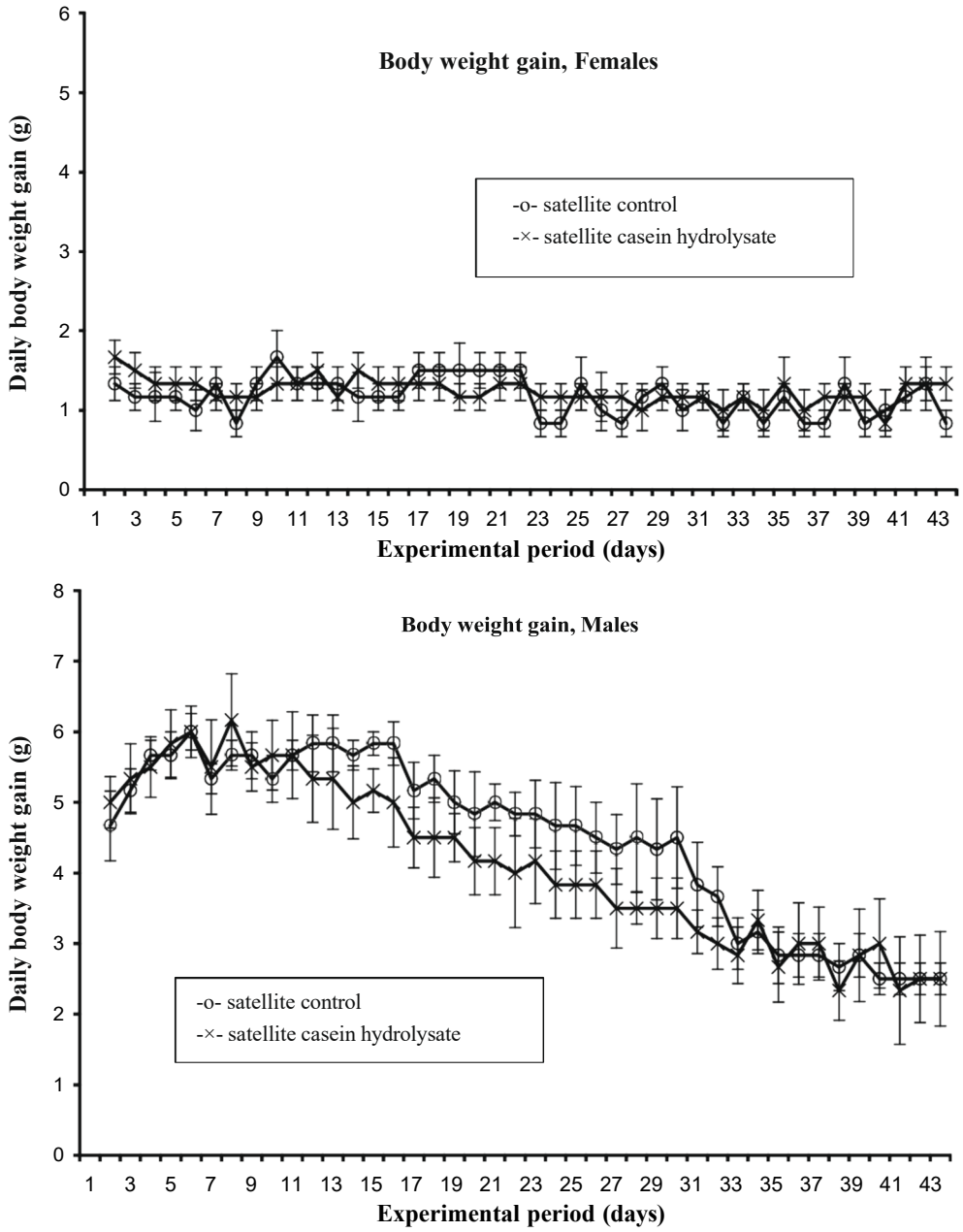


Fig. 3. Daily body weight gain of rats exposed to repeated (4 weeks) oral doses of Lowpept® at 1000 mg/kg of body weight and moreover observed 14 days after treatment (satellite groups). Mean values, $n$ = 6 animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.

### *2.5. Statistical analysis*

All data are expressed as means ± standard error of the mean (SEM) of 6 determinations (i.e. 6 males and 6 females). Differences between control and treated groups were evaluated with a one-way analysis of variance (ANOVA) followed by Dunnett's test (1995), and differences were considered significant at $P < 0.05$.

## 3. Results

### *3.1. Characterization of the casein hydrolysate and ACE-inhibitory activity*

Table 2 shows the gross composition, protein, moisture, ash and mineral content of the bovine casein hydrolysate (Lowpept®) tested in the toxicity studies. Table 2 also shows the amount of peptides derived from $\alpha_{S1}$-casein, RYLGY

1.52 mg/g and AYFYPEL 2.46 mg/g. The ACE-inhibitory activity of the Lowpept® product expressed as $IC_{50}$ value was 72.1 μg/ml.

### *3.2. Acute oral toxicity in rats*

No abnormal clinical signs, behavioural changes, body weight changes, macroscopic findings, or organ weight changes were observed. All animals survived the 2-week observation period. Body weight data are depicted in Fig. 1. There were no statistical differences in body weights among groups. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted (data not shown). Body weight, daily body weight gain, food and water consumption thus were unaffected by the treatment (single oral dose of 2000 mg/kg of the Lowpept®
product).

The haematological and clinical chemistry parameters assessed 2 weeks after administration of the Lowpept® product as a single oral dose of 2000 mg/kg of body weight were not significantly different compared with those of controls (Tables 3 and 4). No treatment-related changes were noted.

There were no statistical differences in organ weight or tissue:body weight ratios related to the test material (data not shown). The Lowpept® product was not associated with any incidence of macroscopic and microscopic changes. No treatment-related histopathological changes were observed 2 weeks after administration of the Lowpept® product as a single oral dose of 2000 mg/kg of body weight, and histological correlates for the organ weight changes were found. Therefore, Lowpept® has a low order of acute toxicity and the oral lethal dose (LD50) for male and female rats is higher than 2000 mg/kg of body weight.

### *3.3. Repeat dose (4 weeks) oral toxicity in rats*

No mortality was observed. No treatment-related changes in the general condition and external appearance were observed in male and female rats treated with the Lowpept® product at 1000 mg/kg of body weight daily dose. The development of the animals during the experimental period corresponded to their specie and age. There was no significant difference in body weight or body weight gain (Figs. 2 and 3) among groups treated with Lowp- ept® product in comparison to the control groups at any time point of the experimental period. All Lowpept® treated groups consumed
similar amounts of food and water (data not shown) to that of the corresponding control groups.

All haematology data were within normal limits and differences between groups were no observed (Table 5). Clinical chemistry data showed no treatment-related alterations at the end of 4- weeks treatment period (Table 6). Individual values and group mean values were within the physiologic ranges. After 14 days without treatment to detect delayed occurrence of potential toxic effects, there were no treatment-related changes in haematological and clinical test parameters (data shown in Tables 5 and 6, satellite control group or Group 5 and satellite treated group or Group 6).

The necropsy performed on day 29 after the last dose of the Lowpept® product (Group 4) and on day 42 after 14 days without any treatment (Group 6) did not reveal any gross pathological changes or any differences in organ weights in comparison to the corresponding control groups. Mean organ weights and rate body weight:organ are presented in Table 7. After 4-weeks of treatment, there were no histopathological findings in the organs examined considered to be treatment related in male and female rats (data not shown). There were also no treatment-related histopatholo- gical findings in the satellite treated group (Group 6) (data not shown).

## 4. Discussion

This study represents the first standard toxicology data on a casein hydrolysate derived from cow's milk (Lowpept®). The pro- cessed final Lowpept® product contains two active peptides (1.52 mg/g RYLGY and 2.46 mg/g AYFYPEL), 66.5% total protein, 26.19 mg/g calcium, 1.18 mg/g magnesium, 4.34 mg/g sodium and 24.95 mg/g potassium. The higher content in potassium, com- pare with sodium and magnesium, is due to the neutralization made with KOH after the hydrolysis process. The content of Ca, Na, and K per gram at day of the hydrolysate could correspond to 2.6, 0.3 and 0.5% of the adequate intake for a male adult person of 31–50 years old, respectively; Mg content could correspond to 0.3% of the recommended dietary allowance (31–50 years male adult) (Dietary Reference Intakes: Applications in Dietary Assess- ment, 2000; Otten et al., 2006). Related to the RYLGY and AYFYPEL peptides, it had been previously found that both peptides exerted a significant antihypertensive activity when administered orally at spontaneously hypertensive rats at doses of 5 mg/kg (Contreras et al., 2009). The ACE-inhibitory activity of the Lowpept® product observed in this study and expressed as $IC_{50}$ value was 72.1 μg/ml. This $IC_{50}$ value was comparable to the activity found in other previously reported antihypertensive protein hydrolysates obtained by enzymatic digestion or by fermentation (Miguel et al., 2004; Muguerza et al., 2006; Otte et al., 2007).

Table 5. Haematological parameters in rats after repeated (4 weeks) oral doses of Lowpept® at 1000 mg/kg of body weight.

| Parameters[a] | Group 3 (Control) | | Group 4 (Lowpept® 1000 mg/kg) | | Group 5 (Satellite control) | | Group 6 (Satellite Lowpept® 1000 mg/kg) | |
|---|---|---|---|---|---|---|---|---|
| | Female | Male | Female | Male | Female | Male | Female | Male |
| *4-Weeks repeated dose* | | | | | | | | |
| RBC ($\times 10^6$/l) | 8.73 ± 0.11 | 8.72 ± 0.11 | 8.68 ± 0.07 | 8.84 ± 0.05 | 8.87 ± 0.09 | 8.56 ± 0.09 | 8.58 ± 0.11 | 8.67 ± 0.10 |
| Haemoglobin (g/dl) | 16.40 ± 0.38 | 16.53 ± 0.06 | 15.68 ± 0.12 | 16.88 ± 0.27 | 16.35 ± 0.10 | 16.67 ± 0.30 | 16.10 ± 0.11 | 16.90 ± 0.30 |
| Haematocrit (%) | 46.98 ± 1.12 | 47.08 ± 0.22 | 44.93 ± 0.36 | 47.83 ± 0.90 | 46.78 ± 0.50 | 45.77 ± 0.68 | 45.67 ± 0.37 | 47.12 ± 0.89 |
| MCV (fl) | 52.77 ± 0.39 | 53.00 ± 0.83 | 51.67 ± 0.72 | 52.70 ± 0.20 | 53.52 ± 0.38 | 54.05 ± 0.26 | 53.15 ± 0.41 | 52.82 ± 0.88 |
| MCH (pg) | 18.43 ± 0.11 | 18.67 ± 0.21 | 18.02 ± 0.23 | 18.50 ± 0.10 | 18.67 ± 0.13 | 18.93 ± 0.16 | 18.77 ± 0.14 | 18.47 ± 0.30 |
| MCHC (g/dl) | 34.88 ± 0.07 | 35.13 ± 0.10 | 34.97 ± 0.12 | 35.20 ± 0.13 | 35.02 ± 0.08 | 35.67 ± 0.18 | 35.33 ± 0.15 | 35.52 ± 0.22 |
| RDW (%) | 18.23 ± 0.23 | 18.23 ± 0.30 | 17.62 ± 0.25 | 18.18 ± 0.17 | 18.25 ± 0.20 | 17.98 ± 0.24 | 18.08 ± 0.22 | 19.33 ± 0.74 |
| WBC ($\times 10^3$/l) | 6.86 ± 0.66 | 9.49 ± 0.34 | 7.60 ± 0.48 | 10.71 ± 0.49 | 7.76 ± 0.88 | 9.88 ± 0.67 | 6.52 ± 0.50 | 9.59 ± 0.62 |
| Banded neutrophils ($\times 10^3$/l) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Neutrophils ($\times 10^3$/l) | 1.06 ± 0.10 | 1.59 ± 0.15 | 1.01 ± 0.12 | 1.49 ± 0.13 | 1.04 ± 0.15 | 1.26 ± 0.04 | 0.84 ± 0.04 | 1.14 ± 0.10 |
| Eosinophils ($\times 10^3$/l) | 0.09 ± 0.02 | 0.14 ± 0.02 | 0.11 ± 0.02 | 0.18 ± 0.03 | 0.09 ± 0.02 | 0.17 ± 0.05 | 0.08 ± 0.01 | 0.12 ± 0.02 |
| Lymphocytes ($\times 10^3$/l) | 6.73 ± 0.16 | 7.81 ± 0.16 | 7.78 ± 0.60 | 8.64 ± 0.56 | 7.05 ± 0.56 | 7.51 ± 0.44 | 5.59 ± 0.48 | 7.82 ± 0.62 |
| Monocytes ($\times 10^3$/l) | 0.25 ± 0.02 | 0.31 ± 0.02 | 0.22 ± 0.03 | 0.41 ± 0.05 | 0.30 ± 0.01 | 0.51 ± 0.08 | 0.33 ± 0.04 | 0.40 ± 0.07 |
| Basophils ($\times 10^3$/l) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Platelets ($\times 10^3$/l) | 799.17 ± 31.91 | 798.67 ± 31.97 | 744.00 ± 31.13 | 766.33 ± 26.77 | 853.17 ± 18.83 | 782.17 ± 20.55 | 779.67 ± 29.18 | 847.33 ± 22.86 |
| MPV (fl) | 8.80 ± 0.19 | 8.53 ± 0.05 | 8.47 ± 0.05 | 8.87 ± 0.23 | 8.60 ± 0.12 | 8.58 ± 0.07 | 8.70 ± 0.14 | 8.77 ± 0.15 |
| Prothrombin time (s) | 17.25 ± 0.25 | 17.68 ± 0.39 | 17.52 ± 0.27 | 16.82 ± 0.36 | 17.82 ± 0.41 | 18.57 ± 0.35 | 17.28 ± 0.50 | 18.83 ± 0.53 |
| Thromboplastin partial time (s) | 27.97 ± 1.82 | 23.68 ± 0.32 | 33.22 ± 1.61 | 23.62 ± 0.83 | 29.00 ± 0.64 | 27.52 ± 1.12 | 29.80 ± 1.76 | 24.93 ± 0.65 |
| Fibrinogen (mg/dl) | 234.00 ± 7.37 | 313.67 ± 33.70 | 196.67 ± 2.76 | 297.17 ± 11.63 | 251.83 ± 11.40 | 322.50 ± 35.96 | 258.83 ± 12.17 | 381.33 ± 10.63 |

Group 3 and Group 4 were sacrificed after treatment on Day 29. Differences between treated group (Group 4) and the control group (Group 3) were not significant. Group 5 and Group 6 were sacrificed on Day 42 after an additional observation period of 14 days without treatment. Differences between satellite treated group (Group 6) and the satellite control group (Group 5) were not significant.

[a] Data are expressed as mean ± SEM ($n$ = 6 animals/sex group).

Table 6. Clinical chemistry in rats after repeated (4 weeks) oral doses of Lowpept® at 1000 mg/kg of body weight.

| Parameters[a] | Group 3 (Control) | | Group 4 (Lowpept® 1000 mg/kg) | | Group 5 (Satellite control) | | Group 6 (Satellite Lowpept® 1000 mg/kg) | |
|---|---|---|---|---|---|---|---|---|
| | Female | Male | Female | Male | Female | Male | Female | Male |
| *4-Weeks repeated dose* | | | | | | | | |
| Glucose (mg/dl) | 103.33 ± 3.14 | 126.33 ± 3.91 | 103.33 ± 3.14 | 120.17 ± 3.76 | 112.17 ± 2.52 | 109.50 ± 0.85 | 102.17 ± 3.77 | 105.33 ± 2.39 |
| Urea nitrogen (mg/dl) | 58.17 ± 18.73 | 34.17 ± 1.22 | 41.17 ± 2.52 | 37.50 ± 2.23 | 40.83 ± 1.35 | 43.67 ± 0.99 | 40.17 ± 1.76 | 39.83 ± 1.85 |
| Creatinine (mg/dl) | 0.68 ± 0.03 | 0.63 ± 0.02 | 0.63 ± 0.02 | 0.67 ± 0.03 | 0.75 ± 0.02 | 0.58 ± 0.03 | 0.72 ± 0.02 | 0.65 ± 0.02 |
| Albumine (g/dl) | 3.97 ± 0.07 | 3.63 ± 0.03 | 3.85 ± 0.04 | 3.63 ± 0.06 | 3.88 ± 0.09 | 3.37 ± 0.03 | 3.72 ± 0.06 | 3.45 ± 0.07 |
| Total protein (g/dl) | 6.93 ± 0.17 | 6.33 ± 0.09 | 6.67 ± 0.09 | 6.55 ± 0.15 | 7.58 ± 0.19 | 6.40 ± 0.06 | 6.83 ± 0.09 | 6.61 ± 0.11 |
| Total bilirubin (mg/dl) | 0.17 ± 0.02 | 0.18 ± 0.02 | 0.13 ± 0.02 | 0.17 ± 0.02 | 0.18 ± 0.02 | 0.25 ± 0.02 | 0.22 ± 0.02 | 0.27 ± 0.03 |
| Calcium (mg/dl) | 12.47 ± 0.14 | 11.92 ± 0.15 | 12.20 ± 0.13 | 11.83 ± 0.18 | 12.63 ± 0.19 | 12.62 ± 0.13 | 12.22 ± 0.26 | 12.48 ± 0.21 |
| Sodium (mEq/l) | 139.00 ± 0.58 | 137.67 ± 1.12 | 137.83 ± 0.48 | 140.67 ± 1.41 | 142.17 ± 0.60 | 141.33 ± 0.76 | 141.50 ± 0.43 | 143.00 ± 1.24 |
| Potassium (mEq/l) | 6.33 ± 0.22 | 6.48 ± 0.14 | 5.95 ± 0.17 | 6.13 ± 0.24 | 6.00 ± 0.34 | 5.98 ± 0.06 | 5.53 ± 0.20 | 5.67 ± 0.30 |
| ASAT (u/l) | 141.17 ± 7.16 | 131.33 ± 2.80 | 151.50 ± 8.54 | 142.17 ± 8.43 | 132.00 ± 2.93 | 140.83 ± 4.22 | 134.50 ± 9.29 | 148.17 ± 2.39 |
| ALAT (u/l) | 47.17 ± 1.17 | 39.17 ± 2.07 | 45.17 ± 1.40 | 43.17 ± 2.47 | 45.33 ± 1.48 | 42.33 ± 3.11 | 38.00 ± 3.53 | 49.17 ± 2.37 |
| Alkaline phosphatase(u/l) | 276.67 ± 19.22 | 427.33 ± 15.80 | 312.67 ± 24.45 | 481.50 ± 21.09 | 196.00 ± 14.77 | 458.67 ± 20.00 | 209.67 ± 17.56 | 468.17 ± 20.84 |
| Triglyceride (mg/dl) | 143.33 ± 11.35 | 179.00 ± 3.00 | 141.17 ± 12.00 | 177.17 ± 9.90 | 140.33 ± 11.35 | 173.50 ± 18.01 | 139.50 ± 12.44 | 171.83 ± 8.52 |
| Cholesterol (mg/dl) | 61.25 ± 1.05 | 58.50 ± 1.67 | 56.17 ± 2.95 | 55.67 ± 2.20 | 72.17 ± 3.05 | 65.83 ± 5.07 | 59.33 ± 3.94 | 69.17 ± 3.34 |
| HDL (mg/dl) | 39.52 ± 1.45 | 38.32 ± 0.90 | 38.03 ± 0.89 | 36.28 ± 2.09 | 39.65 ± 0.50 | 38.33 ± 3.18 | 38.45 ± 1.82 | 42.05 ± 2.90 |
| LDL (mg/dl) | 9.73 ± 1.60 | 7.74 ± 0.71 | 7.65 ± 0.85 | 9.02 ± 1.05 | 7.55 ± 0.80 | 7.72 ± 1.30 | 8.57 ± 0.90 | 9.05 ± 0.97 |
| Lipoprotein A (mg/dl) | <2 | <2 | <2 | <2 | <2 | <2 | <2 | <2 |

Group 3 and Group 4 were sacrificed after treatment on Day 29. Differences between treated group (Group 4) and the control group (Group 3) were not significant. Group 5 and Group 6 were sacrificed on Day 42 after an additional observation period of 14 days without treatment. Differences between satellite treated group (Group 6) and the satellite control group (Group 5) were not significant.

[a] Data are expressed as mean ± SEM ($n$ = 6 animals/sex group).

Table 7. Mean organ weights and rate body weight/organ in rats after repeated (4 weeks) oral doses of Lowpept® at 1000 mg/kg of body weight.

| Parameters[a] | Group 3 (Control) | | Group 4 (Lowpept® 1000 mg/kg) | | Group 5 (Satellite control) | | Group 6 (Satellite Lowpept® 1000 mg/kg) | |
|---|---|---|---|---|---|---|---|---|
| | Female | Male | Female | Male | Female | Male | Female | Male |
| *4-Weeks repeated dose* | | | | | | | | |
| Body weight (g) | 238.83 ± 7.06 | 328.00 ± 9.63 | 236.00 ± 7.31 | 342.50 ± 3.00 | 237.67 ± 2.75 | 395.67 ± 3.25 | 242.50 ± 1.94 | 379.67 ± 10.85 |
| Increase body weight (g) | 48.00 ± 5.08 | 110.67 ± 9.42 | 46.67 ± 6.99 | 123.17 ± 3.23 | 47.33 ± 0.67 | 183.33 ± 3.65 | 50.33 ± 1.80 | 169.00 ± 11.19 |
| Brain weight (g) | 1.82 ± 0.03 | 1.92 ± 0.02 | 1.84 ± 0.03 | 1.88 ± 0.03 | 1.80 ± 0.02 | 1.94 ± 0.04 | 1.80 ± 0.03 | 1.90 ± 0.04 |
| Rate body weight/brain (%) | 0.77 ± 0.02 | 0.59 ± 0.02 | 0.78 ± 0.03 | 0.55 ± 0.01 | 0.76 ± 0.01 | 0.49 ± 0.01 | 0.74 ± 0.01 | 0.50 ± 0.02 |
| Thymus weight (g) | 0.40 ± 0.05 | 0.70 ± 0.01 | 0.49 ± 0.03 | 0.63 ± 0.04 | 0.43 ± 0.01 | 0.53 ± 0.05 | 0.44 ± 0.01 | 0.61 ± 0.05 |
| Rate body weight/thymus (%) | 0.17 ± 0.02 | 0.20 ± 0.01 | 0.21 ± 0.01 | 0.18 ± 0.01 | 0.18 ± 0.004 | 0.13 ± 0.01 | 0.18 ± 0.01 | 0.16 ± 0.01 |
| Heart weight (g) | 0.64 ± 0.02 | 0.94 ± 0.01 | 0.70 ± 0.02 | 0.93 ± 0.01 | 0.70 ± 0.01 | 0.93 ± 0.08 | 0.70 ± 0.02 | 0.97 ± 0.02 |
| Rate body weight/heart (%) | 0.27 ± 0.01 | 0.29 ± 0.01 | 0.30 ± 0.01 | 0.27 ± 0.005 | 0.29 ± 0.005 | 0.23 ± 0.02 | 0.29 ± 0.01 | 0.26 ± 0.01 |
| Right lung weight (g) | 0.55 ± 0.03 | 0.76 ± 0.03 | 0.60 ± 0.03 | 0.75 ± 0.01 | 0.57 ± 0.01 | 0.87 ± 0.02 | 0.61 ± 0.03 | 0.83 ± 0.08 |
| Rate body weight/right lung (%) | 0.23 ± 0.02 | 0.23 ± 0.01 | 0.25 ± 0.01 | 0.22 ± 0.005 | 0.24 ± 0.005 | 0.22 ± 0.004 | 0.25 ± 0.01 | 0.22 ± 0.02 |
| Left lung weight (g) | 0.38 ± 0.03 | 0.45 ± 0.01 | 0.33 ± 0.02 | 0.43 ± 0.02 | 0.33 ± 0.01 | 0.47 ± 0.01 | 0.34 ± 0.01 | 0.49 ± 0.07 |
| Rate body weight/left lung (%) | 0.16 ± 0.01 | 0.14 ± 0.004 | 0.14 ± 0.005 | 0.13 ± 0.004 | 0.14 ± 0.004 | 0.12 ± 0.003 | 0.14 ± 0.01 | 0.13 ± 0.02 |
| Liver weight (g) | 8.27 ± 0.42 | 12.34 ± 0.20 | 8.82 ± 0.56 | 12.35 ± 0.27 | 7.21 ± 0.17 | 13.79 ± 0.18 | 6.85 ± 0.22 | 13.15 ± 0.35 |
| Rate body weight/liver (%) | 3.46 ± 0.13 | 3.78 ± 0.11 | 3.72 ± 0.14 | 3.60 ± 0.05 | 3.04 ± 0.09 | 3.49 ± 0.05 | 2.82 ± 0.08 | 3.48 ± 0.13 |
| Spleen weight (g) | 0.51 ± 0.02 | 0.74 ± 0.01 | 0.57 ± 0.05 | 0.75 ± 0.04 | 0.52 ± 0.02 | 0.80 ± 0.01 | 0.50 ± 0.02 | 0.76 ± 0.05 |
| Rate body weight/spleen (%) | 0.21 ± 0.01 | 0.23 ± 0.01 | 0.24 ± 0.02 | 0.22 ± 0.01 | 0.22 ± 0.01 | 0.20 ± 0.004 | 0.21 ± 0.01 | 0.20 ± 0.02 |
| Pancreas weight (g) | 0.30 ± 0.05 | 0.47 ± 0.04 | 0.24 ± 0.02 | 0.49 ± 0.20 | 0.29 ± 0.01 | 0.33 ± 0.02 | 0.23 ± 0.03 | 0.34 ± 0.02 |
| Rate body weight/pancreas (%) | 0.12 ± 0.02 | 0.14 ± 0.01 | 0.10 ± 0.01 | 0.14 ± 0.05 | 0.12 ± 0.01 | 0.08 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 |
| Right kidney weight (g) | 0.75 ± 0.02 | 1.13 ± 0.02 | 0.70 ± 0.05 | 1.12 ± 0.03 | 0.70 ± 0.02 | 1.11 ± 0.03 | 0.67 ± 0.01 | 1.11 ± 0.03 |
| Rate body weight/right kidney (%) | 0.31 ± 0.01 | 0.35 ± 0.01 | 0.30 ± 0.01 | 0.33 ± 0.01 | 0.30 ± 0.01 | 0.28 ± 0.01 | 0.27 ± 0.01 | 0.29 ± 0.01 |
| Left kidney weight (g) | 0.73 ± 0.02 | 1.09 ± 0.02 | 0.74 ± 0.05 | 1.08 ± 0.03 | 0.71 ± 0.02 | 1.10 ± 0.01 | 0.68 ± 0.01 | 1.09 ± 0.05 |
| Rate body weight/left kidney (%) | 0.31 ± 0.01 | 0.33 ± 0.005 | 0.31 ± 0.01 | 0.31 ± 0.01 | 0.30 ± 0.01 | 0.28 ± 0.003 | 0.28 ± 0.003 | 0.29 ± 0.01 |
| Right adrenal gland weight (g) | 0.05 ± 0.003 | 0.04 ± 0.002 | 0.05 ± 0.004 | 0.04 ± 0.005 | 0.03 ± 0.003 | 0.04 ± 0.004 | 0.04 ± 0.003 | 0.03 ± 0.001 |
| Rate body weight/right adrenal gland (%) | 0.02 ± 0.001 | 0.01 ± 0.001 | 0.02 ± 0.002 | 0.01 ± 0.001 | 0.01 ± 0.001 | 0.01 ± 0.001 | 0.01 ± 0.001 | 0.01 ± 0.00004 |
| Left adrenal gland weight (g) | 0.05 ± 0.004 | 0.04 ± 0.002 | 0.06 ± 0.01 | 0.04 ± 0.004 | 0.04 ± 0.002 | 0.03 ± 0.004 | 0.04 ± 0.002 | 0.04 ± 0.004 |
| Rate body weight/left adrenal gland (%) | 0.02 ± 0.002 | 0.01 ± 0.001 | 0.02 ± 0.002 | 0.01 ± 0.001 | 0.02 ± 0.001 | 0.01 ± 0.001 | 0.02 ± 0.001 | 0.01 ± 0.001 |
| Right testicle weight (g) | | 1.76 ± 0.04 | | 1.68 ± 0.02 | | 1.79 ± 0.05 | | 1.64 ± 0.05 |
| Rate body weight/right testicle (%) | | 0.54 ± 0.02 | | 0.49 ± 0.005 | | 0.45 ± 0.01 | | 0.44 ± 0.02 |
| Left testicle weight (g) | | 1.77 ± 0.03 | | 1.68 ± 0.04 | | 1.79 ± 0.05 | | 1.68 ± 0.05 |
| Rate body weight/left testicle (%) | | 0.54 ± 0.02 | | 0.49 ± 0.01 | | 0.45 ± 0.01 | | 0.44 ± 0.02 |
| Bone marrow weight (g) | 0.08 ± 0.01 | 0.08 ± 0.004 | 0.09 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.005 | 0.08 ± 0.02 | 0.09 ± 0.02 | 0.09 ± 0.01 |
| Rate body weight/bone marrow (%) | 0.03 ± 0.003 | 0.02 ± 0.001 | 0.04 ± 0.004 | 0.02 ± 0.003 | 0.03 ± 0.002 | 0.02 ± 0.004 | 0.04 ± 0.01 | 0.02 ± 0.004 |

Group 3 and Group 4 were sacrificed after treatment on Day 29. Differences between treated group (Group 4) and the control group (Group 3) were not significant. Group 5 and Group 6 were sacrificed on Day 42 after an additional observation period of 14 days without treatment. Differences between satellite treated group (Group 6) and the satellite control group (Group 5) were not significant.

[a] Data are expressed as mean ± SEM ($n$ = 6 animals/sex group).

For confirmation of the safety of Lowpept® product, the present work assessed its acute and repeated dose (4-weeks) oral toxicity. In the present study, a single oral limit dose of 2000 mg/kg of the Lowpept® product was well tolerated by both male and female rats (limit test). A single dose of 2000 mg/kg of body weight (equivalent to 8 mg RYLGY plus AYFYPEL per kg of body weight) did not result in any observable adverse effects or mortality in both rat sexes and there is no evidence of specific target organs of toxicity. Daily oral administration of 1000 mg Lowpept® /kg of body weight (equivalent to 4 mg RYLGY plus AYFYPEL per kg of body weight per day) for 4 consecutive weeks did not cause mortality, changes in body weight, body weight gain or food consumption. No haematological or clinical pathologic alterations were noted. Both gross and histo-pathological examinations did not reveal any treatment-related changes. The no-observed-adverse-effect level in this repeated dose (4 weeks) oral toxicity study was the dose tested, i.e. 1000 mg/kg. This finding provides the basis for the selection of doses for use in long-term toxicity studies.

Based on the findings from this repeated dose (4-weeks) oral toxicity study, there were no indications of toxicological effects in rats treated orally at 1000 mg Lowpept®/kg of body weight.

Therefore, a logical conclusion from this study is that the Lowpept® product was well tolerated in daily dose at 1000 mg/kg of body weight for 4 weeks in the rat and there was no evidence for systemic toxicity.

The safety of casein hydrolysate was expected taken in account the nature of the substrate (casein) and the proteolytic enzyme employed in its manufacture (pepsin). Casein hydrolysates have a long history of safe use in humans. They have widely been used in infant formulas and infant products for allergic children for more than 60 years. Other applications of milk protein hydrolysates are found in clinical nutrition and sports nutrition (Schaafsma, 2009). Pepsin is a well known proteolytic enzyme with many applications in food manufacture. It is extensively used in cheese production and to modify functional properties of other food proteins of plant of animal origin. In fact, during cheese manufacture and cheese

ripening, a large number of peptides of variable length are released. With the recognition that specific peptides derived from food proteins may exert beneficial effects in the organism, it has been found that many traditional products such as cheese or fermented milk can be a rich source of these bioactive peptides. An extensively work has been performed to quantify the amount of the antihypertensive peptides IPP and VPP by HPLC-MS. Surprisingly, it has been found that the natural concentration of IPP and VPP in hard cheeses may even exceed the dose of these peptides given in functional foods in clinical studies (Bütikofer et al., 2008). Similarly, the antihypertensive peptides contained in casein hydrolysate share high homology with others previously found in cheese or in simulated gastrointestinal digestions of milk products. Peptides with sequences YFYPEL and YPEL, corresponding to $a_{S1}$-CN $f$ (144–149) and $a_{S1}$-CN $f$ (146–149), are released after simulated gastrointestinal digestion of infant formulas (Hernández-Ledesma et al., 2004, 2007). Peptides homologous to RYLGY, concretely ERYLGY and RYLG had previously been described in 8-month-old Manchego cheese and a digestion of an infant formula, respectively (Gómez-Ruiz et al., 2004; Hernández-Ledesma et al., 2004).

In conclusion, the present study has evaluated the potential acute and repeated dose (4 weeks) oral toxicity of a casein hydrolysate containing the antihypertensive peptides RYLGY and AYFYPEL, two sequences that correspond to $a_{S1}$-casein $f$ (90–94) (RYLGY) and $a_{S1}$-casein $f$ (143–149) (AYFYPEL). Based on the present results, the maximal oral dose of Lowpept® product investigated in the 4-weeks repeated dose (1000 mg/kg per day, equivalent to 4 mg RYLGY plus AYFYPEL per kg of body weight per day) study did not show any difference among controls and treated animals, thus representing a dose level of non-observable toxic effects (NOAEL). Overall, no Lowpept®-related toxicity has been observed after single oral limit dose (2000 mg/kg equivalent to 8 mg RYLGY plus AYFYPEL per kg of body weight) suggesting a low potential oral toxicity with promising antihypertensive effects.

Nevertheless, further studies including larger doses and longer periods of treatment should be conducted before its use as a dietary supplement in humans.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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