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ORIGINAL ARTICLE

Safety of long-term dietary supplementation with L-arginine in rats

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Abstract This study was conducted with rats to determine the safety of long-term dietary supplementation with L-arginine. Beginning at 6 weeks of age, male and female rats were fed a casein-based semi-purified diet containing 0.61 % L-arginine and received drinking water containing L-arginine-HCl (0, 1.8, or 3.6 g L-arginine/kg body-weight/day; $n = 10$/group). These supplemental doses of L-arginine were equivalent to 0, 286, and 573 mg L-arginine/kg body-weight/day, respectively, in humans. After a 13-week supplementation period, blood samples were obtained from rats for biochemical analyses. Supplementation with L-arginine increased plasma concentrations of arginine, ornithine, proline, homoarginine, urea, and nitric oxide metabolites without affecting those for lysine, histidine, or methylarginines, while reducing plasma concentrations of ammonia, glutamine, free fatty acids, and triglycerides. L-Arginine supplementation enhanced protein gain and reduced white-fat deposition in the body. Based on general appearance, feeding behavior, and physiological parameters, all animals showed good health during the entire experimental period; Plasma concentrations of all measured hormones (except leptin) did not differ between control and arginine-supplemented rats. L-Arginine supplementation reduced plasma levels of leptin. Additionally, L-arginine supplementation increased L-arginine:glycine amidinotransferase activity in kidneys but not in the liver or small intestine, suggesting tissue-specific regulation of enzyme expression by L-arginine. Collectively, these results indicate that dietary supplementation with L-arginine (e.g., 3.6 g/kg body-weight/day) is safe in rats for at least 91 days. This dose is equivalent to 40 g L-arginine/kg body-weight/day for a 70-kg person. Our findings help guide clinical studies to determine the safety of long-term oral administration of L-arginine to humans.

Keywords Arginine · Humans · Nutrition · Safety · Swine

Abbreviations

ADMA	Asymmetrical dimethylarginine
AGAT	L-Arginine:glycine amidinotransferase
Arg	L-Arginine
BW	Body weight
hArg	L-Homoarginine
HPLC	High-performance liquid chromatography
NMMA	N^G-monomethylarginine
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitrate plus nitrite
SDMA	Symmetrical dimethylarginine

Introduction

L-Arginine is synthesized in virtually all cell types from L-citrulline (Breuillard et al. 2015; Wu et al. 2007b), which

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is derived from L-glutamine/L-glutamate via pyrroline-5-carboxylate synthase (Blachier et al. 2011; Wu et al. 1994) and from L-proline via proline oxidase (Wu 1997) almost exclusively in enterocytes of most mammals, including humans, rats and pigs. L-Arginine is utilized by multiple metabolic pathways to produce protein, nitric oxide (NO), ornithine (the precursor of polyamines, proline, and glutamate), creatine, and agmatine (Wu and Morris 1998). Additionally, post-translational methylation of arginine residues in protein and its subsequent hydrolysis results in the production of asymmetrical dimethylarginine (ADMA), N^G-monomethylarginine (NMMA), and symmetric dimethylarginine (SDMA). ADMA and NMMA are competitive inhibitors of NO synthases (NOS), whereas SDMA inhibits L-arginine transport by cells (Leiper and Vallance 1999). Although L-arginine was considered in the 1960s to be a substrate for the synthesis of L-homoarginine (hArg) by L-arginine:glycine amidinotransferase (AGAT) in rats (Ryan and Wells 1964; Ryan et al. 1968, 1969), little work had been done to examine mammalian hArg synthesis and catabolism until the 2010s. Emerging evidence shows that hArg may be a biomarker for risk for cardiovascular diseases (Atzler et al. 2015; Kayacelebi et al. 2015; Khalil et al. 2013; Michel 2013; Tsikas and Kayacelebi 2014).

Metabolites produced from L-arginine have enormous metabolic versatility (Blachier et al. 2011; Morris 2007; San Gabriel and Uneyama 2013). For example, NO regulates endothelium-dependent relaxation of blood vessels and energy metabolism in animals (Dai et al. 2013; Gornik and Creager 2004; Wu and Meininger 2009). Second, polyamines are required for the synthesis of DNA and protein as well as the proliferation and differentiation of all cell types (Agostinelli 2014). Third, proline is a major amino acid for collagen synthesis and, therefore, plays a key role in remodeling of the extracellular matrix (Phang and Liu 2012). Fourth, hArg is an inhibitor of L-arginine transport by cells (Greene et al. 1993), arginase (Hrabák et al. 1994), as well as liver and bone alkaline phosphohydrolases (Lin and Fishman 1972). Finally, creatine is an antioxidant and participates in energy metabolism in skeletal muscle and nerves (Brosnan and Brosnan 2007). Thus, there is growing interest in the use of L-arginine in improving health and treating various vascular diseases (Popolo et al. 2014; Wu 2014; Yang et al. 2015).

Although much is known about the biochemistry, physiology, and nutrition of L-arginine, the use of L-arginine as a dietary or beverage supplement has been limited due to the concerns of regulatory agencies, policymakers, and consumers over the safety of its long-term administration in humans (i.e., >2 months). This is due to (1) the lack of clinical data in the literature (e.g., concentrations of metabolites in the plasma) (Cicero and Colletti 2015; Shao and Hathcock 2008); and (2) a possible increase in the risk of adverse cardiovascular events in patients with acute myocardial infarction (Schulman et al. 2006). We have recently reported that long-term (91-day) supplementation of L-arginine does not adversely affect blood chemistry or general health status of pigs (Hu et al. 2015). The present study was conducted with rats to determine their physiological responses to long-term dietary supplementation with graded levels of L-arginine-HCl [i.e., 0, 1.8, and 3.6 g L-arginine/kg body-weight (BW)/day]. Availability of data from two animal models will help to guide future clinical studies (FDA 2005), such as those involving dietary supplementation with high doses of L-arginine to humans.

Materials and methods

L-Arginine-HCl and L-alanine were products of Ajinomoto Inc. (Tokyo, Japan). Their purity was >99.9 %, as analyzed by high-performance liquid chromatography (Wu and Meininger 2008). The sources of other chemicals were the same as described previously (Hou et al. 2015; Hu et al. 2015).

Experiment 1: effects of long-term dietary L-arginine-HCl supplementation on male rats

Animals and diets

Male Sprague–Dawley rats (5 weeks of age) were purchased from Harlan Laboratories (Indianapolis, IN). Upon arrival at the Texas A&M University Kleberg animal facilities, all rats were housed individually in carbonate cages in a temperature-controlled (25 °C) and humidity-controlled (60 % relative humidity) room on a 12-h light:12-h dark cycle. During a 1-week period of adaptation, the animals were fed a regular rodent diet (Product Cat #8604, Harlan Teklad) and had free access to drinking water (double-distilled and deionized water).

At 6 weeks of age, rats were assigned randomly to one of three treatment groups (0, 2.18, or 4.35 g L-arginine-HCl/kg BW/day). These doses of L-arginine-HCl provided 0, 1.8, or 3.6 g L-arginine/kg BW/day, respectively. Isonitrogenous amounts of L-alanine were added to the drinking water (Table 1). The dosages of L-arginine and L-alanine were chosen on the basis of previous studies with rats (Tsubuku et al. 2004; Fu et al. 2005) and pigs (Hu et al. 2015). L-Alanine was used as the isonitrogenous control primarily because of its extensive catabolism in the body, its safety, and its inability to serve as a precursor for endogenous synthesis of arginine (Jobgen et al. 2009a; Kohli et al. 2004). There were 10 rats per group. During the entire period of the experiment, rats were fed a casein-based semi-purified diet containing 4.3 % fat (Jobgen et al.

Table 1 Amounts of L-arginine and L-alanine supplemented to drinking water for rats

L-Arginine-HCl supplemented to drinking water[a] (g/kg BW/day)	Amount of L-arginine equivalent[b] (g/kg BW/day)	L-Alanine supplemented to drinking water[c] (g/kg BW/day)
0	0.00	7.36
2.18	1.80	3.68
4.35	3.60	0.00

[a] Mean water consumption was 87.1, 84.9, and 85.6 ml/kg BW/day for male rats receiving dietary supplementation with 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day via drinking water, respectively. Mean concentrations of L-arginine-HCl in drinking water were 0, 2.57, and 5.08 g/100 ml for male rats in the 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day groups, respectively. Mean water consumption was 109, 106, and 111 ml/kg BW/day for female rats receiving dietary supplementation with 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day, respectively. Mean concentrations of L-arginine-HCl in drinking water were 0, 2.06, and 3.92 g/100 ml for female rats in the 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day groups, respectively. Drinking water was changed daily

[b] The amount of L-arginine (molecular weight: 174.2) was calculated on the basis of the molecular weight (210.7) of L-arginine-HCl

[c] L-Alanine (molecular weight: 89.1) was provided as the isonitrogenous control for L-arginine-HCl. Total supplemental nitrogen was 82.6 mmol/kg BW/day. Mean concentrations of L-alanine in drinking water were 8.45 and 4.33 g/100 ml for male rats in the 0 and 2.18 g L-arginine-HCl/kg BW/day groups, respectively. Mean concentrations of L-alanine in drinking water were 6.75 and 3.47 g/100 ml for female rats in the 0 and 2.18 g L-arginine-HCl/kg BW/day groups, respectively

2009a) and had free access to their respective drinking water (double-distilled and deionized water). This enteral diet contained 0.61 % L-arginine and 0.44 % L-alanine, as analyzed by HPLC (Dai et al. 2014). Concentrations of L-arginine-HCl in drinking water were adjusted according to the volume of water consumed by rats to provide intake of 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day. Mean concentrations of L-arginine-HCl in drinking water were 0, 2.57, and 5.08 g/100 ml for male rats in the 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day groups, respectively.

Because our pilot studies showed that arginine-supplemented rats tended to eat more food than control rats, arginine-supplemented rats were individually pair-fed with rats in the control group (no supplemental L-arginine-HCl) on a kg BW basis to ensure similar intakes of all nutrients (except for arginine and alanine) among the three groups. Body weight, food intake, and water intake of each rat were recorded on a daily basis throughout the study. No spillage of food (pellet form) was noted for any group of rats. Urine glucose and ketone bodies were tested every 4 weeks using Chemstrip.up, as described previously (Wu 1995a).

During the 13th week of arginine supplementation, oxygen consumption, CO_2 production, and energy expenditure were measured between 9:00 and 11:00 AM using a computer-controlled Oxymas instrument (Columbus Instruments, OH, USA), as we described previously (Tekwe et al. 2013). Furthermore, non-invasive tail-cuff measurement of systolic blood pressure was performed, as described by Wu (1995a). After the 13 weeks of arginine supplementation, rats (19 weeks or 133 days of age) were food deprived for 5 h to obtain blood samples (100 μL) from the tail vein using a microhematocrit (Jobgen et al. 2009a) for analyses of serum glucose and amino acids (except for methylarginines and hArg). On the day of euthanasia, after rectal temperatures of the rats were recorded using a thermometer designed for rats, the animals were immediately anesthetized with CO_2 and killed by cervical dislocation. Cardiac blood samples were collected into heparinized tubes and centrifuged immediately to obtain plasma for analyses of lipids, hormones, methylarginines, and hArg, whereas liver, kidney and jejunum samples (approximately 0.5 g) were immediately obtained and placed in liquid nitrogen (Jobgen et al. 2009a). In addition, retroperitoneal, epididymal, subcutaneous (inguinal), and mesenteric adipose tissues, as well as brown adipose tissue (located in the interscapular region), extensor digitorum longus (EDL) and soleus muscles, brain, liver, kidney, and other tissues were dissected and weighed. Intestinal lumen content was removed before the gut was weighed.

Determination of body composition

After tissues of euthanized rats were weighed, the whole body of each animal (including the empty gastrointestinal tract) was homogenized using a Seydelmann Cutter K64 (Strasser; Stuttgart, Germany), as described by Satterfield et al. (2012, 2013). The content of water, crude protein, crude fat, ash, and carbohydrate was determined, as we described (Jobgen et al. 2009a; Wu et al. 1999).

Analysis of amino acids, glucose, creatinine, ammonia, urea, NOx, and enzymes in plasma

For analysis of metabolites, plasma (0.1 ml) was deproteinized with an equal volume of 1.5 M $HClO_4$, followed by addition of 0.05 ml 2 M K_2CO_3 (Wu et al. 1994). Amino acids (including methylarginines and hArg) in the neutralized extract were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde, as described previously (Hou et al. 2015; Wu and Meininger 2008). The integration of chromatographic peaks was performed using the Millenium-32 Software (Waters, Milford, MA, USA). Glucose was determined enzymatically by a spectrophotometric method involving hexokinase and glucose-6-phosphate dehydrogenase (Satterfield et al. 2013). Creatinine was determined using a kit from Sigma Chemicals (St. Louis, MO, USA). Ammonia and urea were

determined using glutamate dehydrogenase and urease plus glutamate dehydrogenase, respectively (Wu 1995b). Nitrate and nitrite (NOx) levels were determined by an HPLC method involving the enzymatic conversion of nitrate into nitrite and the derivatization of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole, as we described previously (Jobgen et al. 2007). Activities of alanine transaminase and aspartate transaminase in plasma were measured as described by Wu et al. (2000), whereas alkaline phosphatase and lactate dehydrogenase activities were determined using assay kits from Sigma Chemicals.

Analysis of free fatty acids, triglycerides, and hormones in plasma

Free fatty acids and triglycerides in plasma were analyzed using assay kits from Wako Chemicals (Richmond, VA, USA), as we previously described (Jobgen et al. 2009a). Plasma insulin, growth hormone, insulin-like growth factor-I, adiponectin, leptin, corticosterone, total triiodothyronine, and total thyroxine were determined using radioimmunoassay kits for rats (Linco, St. Louis, MO), as we previously described (Jobgen et al. 2009a).

Concentrations of hArg and AGAT activity in tissues

To provide an explanation for our observation that plasma concentrations of hArg were increased in arginine-supplemented rats, we determined concentrations of hArg and AGAT activity in the liver and kidney (which are likely major tissues for hArg synthesis in animals), as we described previously (Hou et al. 2015). Because the enteral diet directly provides arginine and lysine to the lumen of the small intestine, we also measured concentrations of hArg and AGAT activity in the jejunum. AGAT activity in tissue homogenates was measured at 37 °C for 15 min in the presence of 80 mM sodium phosphate buffer (pH 7.5) containing 15 mM L-arginine plus 15 mM L-lysine. Under the enzyme assay conditions used, addition of protease inhibitors (Wu 1997) to tissue homogenates had no effect on AGAT activity in the liver, kidney or small intestine.

Experiment 2: effects of long-term dietary L-arginine-HCl supplementation on female rats

Experiment 2 was conducted as Experiment 1, except that female rats were used instead of male rats. Per kg BW, female rats consumed more water than male rats. Thus, concentrations of L-arginine-HCl in drinking water were adjusted according to the volume of water consumption by female rats to provide intake of 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day. Mean concentrations of L-arginine-HCl in drinking water were 0, 2.06, and 3.92 g/100 ml for female rats in the 0, 2.18, and 4.35 g L-arginine-HCl/kg BW/day groups, respectively.

Statistical analyses

Results are expressed as mean ± SEM. Statistical analyses of data were performed by one-way analysis of variance using the General Linear Models procedures (Assaad et al. 2014b). Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison method (Assaad et al. 2014a, b). A probability value ≤ 0.05 was taken to indicate statistical significance.

Results

Overall observations

Feeding behavior, water consumption, hair, general appearance, body-weight gain, rectal temperature, urination, and defecating were normal for all the male and female rats throughout the 13-week period of L-arginine supplementation (Table 2). Rats in all the groups were alert and vivacious. Blood pH did not differ between control and arginine-supplemented rats. Urine tests for glucose and ketone bodies were negative for all rats. No sickness (including diarrhea) or injury was observed for any animals. All internal organs of the male and female rats appeared normal upon gross examination.

Food intakes and water consumption of rats

Dietary supplementation with 1.8 and 3.6 g L-arginine/kg BW/day did not affect ($P > 0.05$) either food intake or water consumption by male (Exp. 1) or female (Exp. 2) rats (Table 2). Food intakes by male and female rats were approximately 45.6 and 49.5 g/kg BW/day, respectively (Table 2). These levels of food intake supplied 278 and 302 mg L-arginine/kg BW/day in the basal diet to male and female rats, respectively. In either male or female rats, arginine supplementation did not affect their water consumption, which was approximately 86 and 109 ml/kg BW/day, respectively (Table 2). Through adjusting L-arginine-HCl concentrations in drinking water, both male and female rats received supplementation of L-arginine in the amounts of 0, 1.8, and 3.6 g/kg BW/day, which were approximately 0, 6, and 12 times the L-arginine intake from the basal diet, respectively. Based on the conversion ratio of 1:0.16 (rats vs. humans; FDA 2005), the human-equivalent doses of the supplemental L-arginine were 0, 286, and 573 mg L-arginine/kg body-weight/day, respectively, or 20, and 40 g L-arginine/day for a 70-kg person, respectively.

Table 2 Effects of dietary supplementation with L-arginine on body weight, food and water intakes, rectal temperature, and blood pH of rats

Variable	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
Initial BW[a] (g)	196 ± 3.3	198 ± 3.6	196 ± 3.5	168 ± 3.8	166 ± 4.0	153 ± 4.2
Final BW[b] (g)	450 ± 7.3	454 ± 7.5	459 ± 7.2	294 ± 5.0	298 ± 5.2	301 ± 5.7
Food intake (g/kg BW/day)	45.6 ± 0.8	45.8 ± 1.1	45.3 ± 0.9	49.6 ± 1.0	49.8 ± 1.3	49.2 ± 1.2
Water intake (ml/kg BW/day)	87.1 ± 1.5	84.9 ± 2.0	85.6 ± 2.3	109 ± 2.4	106 ± 2.8	111 ± 2.7
RT[b] (°C)	36.3 ± 0.02	36.2 ± 0.03	36.3 ± 0.02	36.4 ± 0.03	36.3 ± 0.03	36.4 ± 0.04
Blood pH[b]	7.40 ± 0.01	7.40 ± 0.02	7.40 ± 0.01	7.40 ± 0.02	7.40 ± 0.01	7.40 ± 0.01
sBP[b] (mmHg)	114 ± 1.3[c]	109 ± 1.2[d]	105 ± 1.1[e]	112 ± 1.1[c]	107 ± 1.0[d]	103 ± 1.0[e]

Values are mean ± SEM, $n = 10$/group. Beginning at 6 weeks of age, Sprague–Dawley rats were fed a semi-purified diet (Jobgen et al. 2009a, b) and received drinking water containing supplemental L-arginine-HCl as indicated in Table 1. Except for systolic blood pressure, none of the other measured variables differ among the treatment groups for either males or females ($P > 0.05$)

RT rectal temperature, *sBP* systolic blood pressure

[a] 6 weeks of age

[b] After a 13-week (91-day) period of supplementation

[c,d,e] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

Body weight, whole-body energy expenditure, blood pressure, and body composition of rats

In either male (Exp. 1) or female (Exp. 2) rats, supplementing 1.8 and 3.6 g L-arginine/kg BW/day did not affect ($P > 0.05$) the body weight of the animals (Table 2). However, weights of skeletal muscle, brown adipose tissue, and thymus were increased ($P < 0.05$) but weights of white adipose tissue were decreased ($P < 0.05$) in both male and female rats in response to dietary supplementation with arginine (Table 3). Weights of other tissues did not differ between control and arginine-supplemented rats. L-Arginine supplementation enhanced ($P < 0.05$) oxygen consumption, CO_2 production, and energy expenditure [expressed per kg non-fat mass (Assaad et al. 2014a)] (Table 4), while reducing ($P < 0.05$) systolic blood pressure (Table 2), in both male and female rats. Compared with the control group, supplementing 1.8 and 3.6 g L-arginine/kg BW/day to male or female rats decreased ($P < 0.05$) the percentage of fat and increased ($P < 0.05$) the percentage of protein in the body (Table 5).

Concentrations of amino acids, other metabolites, hormones, and enzymes in the plasma of rats

Plasma concentrations of amino acids and related metabolites in control and arginine-supplemented male or female rats are summarized in Table 6. Dietary supplementation with 1.8 and 3.6 g L-arginine/kg BW/day dose dependently increased ($P < 0.05$) plasma concentrations of arginine, ornithine, proline, hArg, and urea, while reducing plasma concentrations of glutamine, ammonia, free fatty acids, and triglycerides in both males and females. Dietary supplementation with arginine did not affect ($P > 0.05$) concentrations of lysine, histidine, citrulline, ADMA, NMMA, SDMA, other amino acids, creatinine, or glucose in the plasma of male or female rats (Table 6). Concentrations of all measured hormones (except leptin), as well as the activities of alanine transaminase, aspartate transaminase, and lactate dehydrogenase in the plasma did not differ ($P > 0.05$) between control and arginine-supplemented rats (Table 7). L-Arginine supplementation reduced ($P < 0.05$) the concentration of leptin but increased ($P < 0.05$) the activity of alkaline phosphatase in the plasma of both male and female rats in a dose-dependent manner (Table 7).

Concentrations of hArg and AGAT activity in tissues of rats

The concentrations of hArg and AGAT activity were highest in the liver, followed by the kidneys and the small intestine in descending order (Table 8). Supplementing 1.8 and 3.6 g L-arginine/kg BW/day to male and female rats increased ($P < 0.05$) concentrations of hArg and AGAT activity in the kidney, but had no effect ($P > 0.05$) in the liver or the small intestine (Table 8).

Discussion

L-Arginine is an abundant amino acid in animals, representing 14 % of total nitrogen in body protein (Wu et al. 1999; Wu 2014). Besides serving as a major substrate for synthesis of proteins, L-arginine is actively utilized via multiple pathways to generate low-molecular-weight

Table 3 Effects of dietary supplementation with L-arginine on tissue weights of rats

Variable	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
BAT (mg)	702 ± 16^{b}	764 ± 19^{a}	806 ± 21^{a}	484 ± 12^{b}	532 ± 15^{a}	566 ± 17^{a}
Brain (g)	1.82 ± 0.07	1.84 ± 0.08	1.79 ± 0.08	1.26 ± 0.05	1.24 ± 0.06	1.28 ± 0.05
EDL muscle (mg)	173 ± 3.8^{c}	185 ± 4.1^{b}	198 ± 4.3^{a}	118 ± 3.4^{c}	127 ± 3.2^{b}	138 ± 3.6^{a}
Heart (g)	1.60 ± 0.06	1.64 ± 0.06	1.61 ± 0.06	1.05 ± 0.04	1.08 ± 0.04	1.02 ± 0.04
Kidneys (g)	2.75 ± 0.07	2.79 ± 0.08	2.72 ± 0.08	1.80 ± 0.05	1.84 ± 0.06	1.77 ± 0.05
Liver (g)	12.2 ± 0.75	12.5 ± 0.80	12.0 ± 0.72	7.88 ± 0.53	7.93 ± 0.57	7.84 ± 0.60
Lungs (g)	1.77 ± 0.09	1.78 ± 0.08	1.81 ± 0.08	1.20 ± 0.08	1.17 ± 0.07	1.18 ± 0.07
Ovaries (mg)	–	–	–	135 ± 4.1	134 ± 3.9	137 ± 4.4
Pancreas (g)	1.08 ± 0.06	1.12 ± 0.07	1.14 ± 0.07	0.73 ± 0.04	0.75 ± 0.06	0.74 ± 0.05
Small intestine (g)	6.53 ± 0.41	6.68 ± 0.43	6.60 ± 0.38	4.29 ± 0.35	4.31 ± 0.36	4.40 ± 0.38
Large intestine (g)	2.40 ± 0.18	2.52 ± 0.21	2.47 ± 0.22	1.55 ± 0.12	1.63 ± 0.14	1.58 ± 0.14
Soleus muscle (mg)	164 ± 3.6^{c}	177 ± 3.8^{b}	192 ± 4.0^{a}	112 ± 3.3^{c}	123 ± 3.4^{b}	134 ± 3.6^{a}
Spleen (mg)	841 ± 20	853 ± 18	849 ± 21	573 ± 16	580 ± 16	588 ± 18
Stomach (g)	2.27 ± 0.17	2.31 ± 0.15	2.19 ± 0.18	1.59 ± 0.14	1.63 ± 0.13	1.57 ± 0.14
Testes (g)	3.92 ± 0.10	3.85 ± 0.11	3.94 ± 0.13	–	–	–
Thymus (mg)	203 ± 6.8^{b}	226 ± 7.1^{a}	240 ± 7.6^{a}	134 ± 7.5^{b}	157 ± 7.9^{a}	169 ± 8.2^{a}
WAT[A] (g)	12.6 ± 0.93^{a}	7.24 ± 0.58^{b}	4.61 ± 0.32^{c}	8.53 ± 0.61^{a}	4.92 ± 0.40^{b}	3.23 ± 0.25^{c}

Values are mean ± SEM, $n = 10$/group

BAT brown adipose tissue, *EDL* extensor digitorum longus, *WAT* white adipose tissue

[A] Sum of retroperitoneal, epididymal, subcutaneous, and mesenteric white adipose tissues

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

Table 4 Effects of dietary supplementation with L-arginine on energy expenditure in rats

Variable	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
O_2 consumption	1.54 ± 0.012^{c}	1.62 ± 0.013^{b}	1.68 ± 0.013^{a}	1.67 ± 0.010^{c}	1.75 ± 0.012^{b}	1.82 ± 0.013^{a}
CO_2 production	1.38 ± 0.010^{c}	1.46 ± 0.011^{b}	1.52 ± 0.011^{a}	1.49 ± 0.008^{c}	1.58 ± 0.010^{b}	1.65 ± 0.010^{a}
Respiratory quotient	0.896 ± 0.006	0.901 ± 0.007	0.905 ± 0.007	0.894 ± 0.005	0.904 ± 0.006	0.908 ± 0.006
Heat production	7.47 ± 0.058^{c}	7.87 ± 0.061^{b}	8.17 ± 0.062^{a}	8.10 ± 0.047^{c}	8.52 ± 0.054^{b}	8.86 ± 0.056^{a}

Values are mean ± SEM, $n = 10$/group. Data are expressed as L/kg non-fat mass/h for O_2 consumption and CO_2; as kcal/kg non-fat mass/h for heat production, and as vol/vol for respiratory quotient (RQ)

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

bioactive substances (e.g., NO, creatine, polyamines, hArg, and agmatine). Thus, mammals, birds, and fish have high requirements for L-arginine, which may be increased in certain physiological (e.g., pregnancy, lactation, exercise, and exposure to a cold environment) and pathological (e.g., injury, burns, obesity, and diabetes) situations (Wu et al. 2013). In addition, L-arginine can regulate multiple metabolic pathways [e.g., the hepatic urea cycle and tetrahydrobiopterin synthesis (Wu and Morris 1998; Shi et al. 2004)], gene expression [e.g., peroxisome proliferator-activated receptor γ coactivator-1α and AMP-activated protein kinase (Fu et al. 2005; Jobgen et al. 2009b)], and cell signaling pathways [e.g., the mammalian target of rapamycin (mTOR) and focal adhesion kinase (Rhoads and Wu 2009; Yao et al. 2008)]. Thus, L-arginine has been employed in clinical studies to improve cardiovascular function, reduce obesity, and treat a variety of diseases associated with inadequate production of NO (Hurt et al. 2014; Li et al. 2014; Lucotti et al. 2006; Wu et al. 2009). Despite its versatile metabolic functions, the use of L-arginine as a dietary or

Table 5 Effects of dietary supplementation with L-arginine on the body composition of rats

	Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6
Male rats (Exp. 1)			
Water	67.4 ± 0.19	67.6 ± 0.20	67.8 ± 0.20
Crude protein	16.8 ± 0.16[c]	17.4 ± 0.17[b]	17.9 ± 0.17[a]
Crude fat	12.3 ± 0.14[a]	11.5 ± 0.13[b]	10.8 ± 0.12[c]
Minerals	3.21 ± 0.08	3.20 ± 0.09	3.21 ± 0.08
Carbohydrates	0.29 ± 0.02	0.30 ± 0.02	0.29 ± 0.02
Female rats (Exp. 2)			
Water	69.1 ± 0.20	69.3 ± 0.21	69.4 ± 0.21
Crude protein	16.2 ± 0.15[c]	16.7 ± 0.16[b]	17.2 ± 0.16[a]
Crude fat	11.3 ± 0.13[a]	10.6 ± 0.12[b]	10.0 ± 0.12[c]
Minerals	3.12 ± 0.07	3.11 ± 0.07	3.11 ± 0.08
Carbohydrates	0.28 ± 0.02	0.29 ± 0.02	0.29 ± 0.02

Values, expressed as percentage (%), are mean ± SEM, $n = 10$. Rats were euthanized at 133 days of age (91 days after initiation of arginine supplementation) to determine their body composition

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

beverage supplement has been limited due to the concerns of regulatory agencies, policymakers, and consumers over the safety of its long-term supplementation in humans (i.e., >2 months), primarily because of the lack of clinical data (Boger and Bode-Boger 2001; Cicero and Colletti 2015; Cynober 2007; Shao and Hathcock 2008).

Mean arginine intake by the U.S. adult population is ~5 g/day (Flynn et al. 2002). Approximately 40 % of L-arginine in the diet is catabolized in the first pass by the small intestine, and the remaining 60 % (namely, 3 g arginine/day) enters the portal circulation (Castillo et al. 1993; Dai et al. 2011). Results of previous short-term studies indicate the absence of a systematic pattern of adverse effects of oral L-arginine administration in adult humans, which precludes the selection of "No Observed Adverse Effect Level" or "Lowest Observed Adverse Effect Level" as the usual approach to identify a tolerable Upper Level of intake for this dietary supplement (Hayashi 2003; Shao and Hathcock 2008). Thus, investigators have developed a newer method for risk assessment, named the Observed Safe Level (OSL) or the Highest Observed Intake, which is defined as the highest intake level with sufficient evidence of safety (FAO/WHO 2006). In a double-blind, placebo-controlled trial with 16 healthy adult males, oral administration of 20 g L-arginine/day for 4 weeks did not result in any adverse effect as determined by standard clinical chemistry indices (Chin-Dusting et al. 1996). Likewise, healthy adults could tolerate oral administration of 40 g L-arginine/day for 1 week (duration of the study; Beaumier et al. 1995). Similarly, results from other trials indicated no side effects of oral administration of 21 and 42 g L-arginine/day to patients with hypercholesterolemia (Clarkson et al. 1996) and cystic fibrosis (Grasemann et al. 2005) for 4 and 6 weeks, respectively. Based on these findings, an OSL value for oral administration of L-arginine to healthy adults has been suggested to be 20 g/day (Shao and Hathcock 2008). However, the published studies with healthy subjects involved a short duration of L-arginine supplementation (1 to 4 weeks) and a very small number of subjects (5–16) (Beaumier et al. 1995; Chin-Dusting et al. 1996). These concerns limit our confidence in the 20 g/day dose as the OSL value for oral administration of L-arginine to healthy adults and underscore the need for larger and longer studies. To date, it is unknown how much dietary arginine can be tolerated by humans for a prolonged period of time (Cicero and Colletti 2015; Evans et al. 2004; McKnight et al. 2010; McNeal et al. 2010). This question can only be answered by doing clinical trials with humans. However, such studies must be based first on long-term animal experiments.

Data from animal studies are much needed before clinical trials are started with humans. We reported that healthy adult pigs and rats can tolerate large amounts of supplemental L-arginine, which are at least 0.21 g/kg body-weight/day (Mateo et al. 2007, 2008) and 2.1 g/kg body-weight/day (Jobgen et al. 2009a, b) for 84 days, respectively (Wu et al. 2007a). Based on standard hematology and clinical chemistry tests, growth, general health status, plasma concentrations of amino acids, other metabolites, hormones (e.g., insulin, growth hormone, and IGF-1), as well as plasma activities of alanine transaminase, aspartate transaminase, and lactate dehydrogenase (indicators of integrity of cells, particularly hepatocytes), we found that dietary supplementation with L-arginine (315, 473, and 630 mg/kg BW/day) was safe in pigs for at least 91 days (Hu et al. 2015). These doses of supplemental arginine are equivalent to 286, 430, and 573 mg L-arginine/kg BW/day, respectively, in humans (Hu et al. 2015). Likewise, Tsubuku et al. (2004) reported that adult male rats can tolerate at least 3.6 g L-arginine/kg BW/day for 13 weeks. Similar results from measurements of various physiological parameters were obtained in the present student involving both male and female rats. Based on the conversion factor adopted by the Food and Drug Administration of the United States (2005), the human-equivalent doses of the supplemental L-arginine from our rat study (1.8 and 3.6 g L-arginine/kg BW/day) were equivalent to 20 and 40 g L-arginine/day for a 70-kg person, respectively.

Dietary supplementation with L-arginine increased plasma concentrations of arginine, ornithine, and proline, while reducing plasma concentrations of ammonia, glutamine, free fatty acids, and triglycerides without any adverse effect on plasma concentrations of other proteinogenic amino acids (e.g., lysine, histidine, glycine,

Table 6 Effects of dietary supplementation with L-arginine on concentrations of amino acids and other metabolites in the plasma of rats

Variable	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
Alanine	731 ± 10^{a}	622 ± 13^{b}	540 ± 11^{c}	750 ± 14^{a}	639 ± 16^{b}	544 ± 12^{c}
Arginine	204 ± 5^{c}	286 ± 6^{b}	379 ± 8^{a}	220 ± 6^{c}	291 ± 8^{b}	383 ± 9^{a}
Asparagine	46 ± 2	48 ± 3	49 ± 3	50 ± 3	49 ± 3	52 ± 4
Aspartate	40 ± 2	41 ± 2	42 ± 2	43 ± 2	42 ± 2	44 ± 3
Citrulline	74 ± 3	76 ± 4	72 ± 4	71 ± 4	73 ± 5	70 ± 4
Glutamate	94 ± 4^{b}	102 ± 5^{ab}	112 ± 5^{a}	91 ± 3^{b}	98 ± 4^{ab}	106 ± 4^{a}
Glutamine	617 ± 11^{a}	564 ± 8^{b}	520 ± 6^{c}	625 ± 13^{a}	577 ± 10^{b}	538 ± 7^{c}
Glycine	401 ± 9	393 ± 10	406 ± 10	448 ± 11	437 ± 8	452 ± 9
Histidine	71 ± 4	70 ± 3	74 ± 4	66 ± 3	64 ± 5	69 ± 4
Isoleucine	119 ± 6	124 ± 8	120 ± 8	128 ± 8	133 ± 9	129 ± 7
Leucine	187 ± 8	191 ± 10	182 ± 8	194 ± 10	198 ± 11	186 ± 10
Lysine	219 ± 13	224 ± 15	213 ± 15	237 ± 12	246 ± 14	230 ± 12
Methionine	91 ± 4	93 ± 5	92 ± 4	97 ± 5	95 ± 6	94 ± 5
Ornithine	63 ± 3^{c}	82 ± 4^{b}	104 ± 5^{a}	69 ± 3^{c}	87 ± 5^{b}	08 ± 6^{a}
Phenylalanine	68 ± 2	66 ± 3	70 ± 3	66 ± 3	64 ± 4	67 ± 4
Proline	304 ± 10^{c}	368 ± 13^{b}	426 ± 15^{a}	314 ± 11	359 ± 12	413 ± 12
Serine	339 ± 13	350 ± 16	344 ± 15	318 ± 15	337 ± 18	330 ± 16
Taurine	423 ± 19	446 ± 23	418 ± 22	447 ± 21	461 ± 25	435 ± 24
Threonine	390 ± 11	385 ± 12	396 ± 12	402 ± 13	408 ± 16	414 ± 15
Tryptophan	94 ± 3	91 ± 4	90 ± 4	98 ± 4	94 ± 4	93 ± 5
Tyrosine	146 ± 9	152 ± 10	155 ± 12	142 ± 11	146 ± 12	152 ± 11
Valine	251 ± 16	249 ± 14	246 ± 16	263 ± 18	255 ± 17	253 ± 17
Creatinine	56.8 ± 3.4	57.2 ± 3.7	57.4 ± 3.5	58.0 ± 3.6	58.6 ± 4.0	59.0 ± 3.9
Homoarginine	2.04 ± 0.03^{c}	2.27 ± 0.03^{b}	2.53 ± 0.04^{a}	2.09 ± 0.03^{c}	2.31 ± 0.04^{b}	2.68 ± 0.04^{a}
ADMA	0.85 ± 0.04	0.87 ± 0.05	0.86 ± 0.05	0.86 ± 0.05	0.86 ± 0.06	0.87 ± 0.05
NMMA	1.06 ± 0.06	1.12 ± 0.07	1.08 ± 0.06	1.04 ± 0.07	1.06 ± 0.06	1.10 ± 0.06
SDMA	0.94 ± 0.05	0.96 ± 0.06	0.95 ± 0.06	0.92 ± 0.06	0.94 ± 0.07	0.93 ± 0.05
Ammonia	101 ± 6.20^{a}	83.6 ± 5.12^{b}	68.2 ± 4.93^{c}	104 ± 6.84	82.1 ± 6.37	66.5 ± 5.40
Urea	2.45 ± 0.11^{c}	2.86 ± 0.13^{b}	3.39 ± 0.15^{a}	2.27 ± 0.12^{c}	2.63 ± 0.14^{b}	3.11 ± 0.16^{a}
NOx	22.0 ± 0.75^{c}	27.4 ± 0.82^{b}	34.6 ± 0.90^{a}	24.8 ± 0.72^{c}	28.6 ± 0.79^{b}	35.8 ± 0.96^{a}
Glucose	6.04 ± 0.06^{a}	5.82 ± 0.05^{b}	5.70 ± 0.05^{b}	5.95 ± 0.05^{a}	5.74 ± 0.05^{b}	5.61 ± 0.06^{b}
Triglycerides	646 ± 25^{a}	560 ± 21^{b}	501 ± 18^{c}	395 ± 16^{a}	332 ± 13^{b}	282 ± 10^{c}
Cholesterol	2.78 ± 0.16	2.84 ± 0.15	2.72 ± 18	2.89 ± 0.18	2.75 ± 0.20	2.91 ± 0.22
Free fatty acids	611 ± 39^{a}	572 ± 36^{ab}	558 ± 31^{b}	426 ± 18^{a}	387 ± 16^{ab}	354 ± 13^{b}

Data are mean ± SEM, $n = 10$/group. Blood samples were obtained from rats 5 h after they were food deprived, at 133 days of age (91 days after initiation of arginine supplementation). Values are expressed as mM for urea, glucose, and cholesterol. Values for all other variables are expressed as μM

NOx Nitrate plus nitrite

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

tryptophan, and methionine) in male or female rats (Table 6). This is consistent with the beneficial roles for L-arginine in activating the hepatic urea cycle and enhancing muscle protein synthesis in mammals (Wu and Morris 1998; Yao et al. 2008), while stimulating the oxidation of fatty acids to water and CO_2 and inhibiting the synthesis of both long-chain fatty acids and triglycerides in a tissue-specific manner (McKnight et al. 2010; Wu et al. 2012). Accordingly, L-arginine-supplemented rats had a higher rate of energy expenditure (Table 4) and gained more protein but less fat in the body (Table 5), as compared with the control group. In addition, our results indicate that dietary supplementation with 1.8 and 3.6 g L-arginine/kg BW/day does not cause either antagonism among basic amino acids or an

Table 7 Effects of dietary supplementation with L-arginine on concentrations of hormones in the plasma of rats

Variables in plasma	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
Insulin	237 ± 12	248 ± 13	256 ± 13	219 ± 14	230 ± 17	238 ± 15
Growth hormone	5.22 ± 0.41	5.34 ± 0.46	5.39 ± 0.48	7.63 ± 0.53	7.81 ± 0.61	7.95 ± 0.58
IGF-1	728 ± 63	737 ± 66	751 ± 69	606 ± 59	614 ± 54	631 ± 56
Corticosterone	62.9 ± 5.03	60.6 ± 5.37	61.3 ± 5.19	85.1 ± 6.22	88.7 ± 7.35	83.4 ± 6.94
Adiponectin	4.24 ± 0.31	4.18 ± 0.33	4.05 ± 0.30	5.63 ± 0.44	5.79 ± 0.48	5.52 ± 0.41
Leptin	6.15 ± 0.41[a]	5.09 ± 0.28[b]	4.15 ± 0.24[c]	5.02 ± 0.36[a]	4.28 ± 0.22[b]	3.50 ± 0.19[c]
Total T_3	748 ± 52	733 ± 49	741 ± 44	863 ± 64	850 ± 61	874 ± 68
Total T_4	35.0 ± 2.7	36.6 ± 3.0	34.7 ± 2.8	37.1 ± 3.2	38.4 ± 3.4	36.6 ± 3.0
ALT	52.2 ± 3.0	50.8 ± 2.7	51.3 ± 3.2	54.3 ± 2.6	52.9 ± 3.1	53.6 ± 3.3
AST	58.4 ± 3.6	59.2 ± 3.4	57.7 ± 3.9	59.7 ± 4.2	61.2 ± 3.6	60.1 ± 3.5
ALP	125 ± 5.3[c]	141 ± 5.8[b]	160 ± 6.1[a]	120 ± 4.7[c]	138 ± 5.0[b]	154 ± 5.5[a]
LDH	326 ± 23	319 ± 25	314 ± 20	317 ± 19	311 ± 21	322 ± 18

Values are mean ± SEM, $n = 10$/group. Blood samples were obtained from rats 5 h after they were food deprived, at 133 days of age (91 days after initiation of arginine supplementation). Data are expressed as pM for insulin; as μg/L for adiponectin, leptin, growth hormone, insulin-like growth factor-I (IGF-1), and corticosterone; as ng/L for total triiodothyronine (T_3) and total thyroxine (T_4); and as U/L for alanine transaminase activity (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH)

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

Table 8 Effects of dietary L-arginine supplementation on homoarginine concentration and AGAT activity in tissues of rats

Tissue	Male rats (Exp. 1)			Female rats (Exp. 2)		
	Supplemental arginine (g/kg BW/day)			Supplemental arginine (g/kg BW/day)		
	0	1.8	3.6	0	1.8	3.6
Homoarginine concentration (nmol/g wet tissue)						
Kidney	67.5 ± 1.82[c]	75.7 ± 1.88[b]	83.3 ± 1.93[a]	66.2 ± 1.71[c]	74.4 ± 1.80[b]	81.6 ± 1.85[a]
Liver	78.2 ± 2.04	79.4 ± 2.16	78.6 ± 2.30	77.4 ± 1.86	76.9 ± 1.95	79.1 ± 2.12
Small intestine	18.3 ± 0.56	17.6 ± 0.68	18.0 ± 0.61	16.9 ± 0.60	17.5 ± 0.63	17.2 ± 0.66
AGAT activity (nmol homoarginine/g wet tissue/min)						
Kidney	10.9 ± 0.65[c]	14.2 ± 0.91[b]	17.7 ± 1.08[a]	11.3 ± 0.78[c]	15.5 ± 0.96[b]	18.3 ± 1.17[a]
Liver	214 ± 7.2	220 ± 8.4	226 ± 9.1	206 ± 8.0	212 ± 9.3	217 ± 9.6
Small intestine	4.21 ± 0.31	4.15 ± 0.33	4.33 ± 0.35	4.15 ± 0.33	4.28 ± 0.38	4.36 ± 0.39

Values are mean ± SEM, $n = 10$/group

[a,b,c] Within a row for the male or female group, means with different superscript letters differ ($P < 0.05$)

imbalance between amino acids in male or female rats. The observation that the plasma activity of alkaline phosphatase (an indicator of its release by both the liver and bone) was higher in arginine-supplemented rats than in the control group, as reported previously for pigs (Hu et al. 2015), may suggest a stimulatory effect of L-arginine on bone growth. Collectively, our results indicate that dietary supplementation with L-arginine (at least 3.6 g/kg BW/day) is safe in both male and female rats for at least 91 days. Our findings help guide clinical studies to determine the safety of long-term oral administration of L-arginine to humans.

Finally, plasma concentrations of methylarginines and hArg in control and arginine-supplemented rats warrant comments. Because the experimental diet did not contain these amino acids, changes in their plasma levels are determined by the balance between production and utilization/excretion (Wu 2013). Although L-arginine had long been identified to be the precursor of ADMA, NMMA, and SDMA (Leiper and Vallance 1999), as well as hArg (Davids et al. 2012; Ryan et al. 1969) in animals, little is known about effects of arginine supplementation on plasma concentrations of these amino acids or about the nutritional regulation of their

synthesis in animals or humans. We found that supplementing 1.8 and 3.6 g arginine/kg BW/day for 13 weeks did not affect the concentrations of ADMA, NMMA, and SDMA in the plasma of male or female rats (Table 6). Thus, substantial increases in the ratios of arginine to ADMA, NMMA, and SDMA can overcome inhibitory effects of the methylarginines on NOS and the uptake of arginine by cells (Tsikas et al. 2000), thereby promoting NO production by various tissues and cells (including vascular endothelial cells) and reducing blood pressure (Table 2) in both male and female rats. Similarly, hArg concentrations or AGAT activities in the liver or the small intestine did not differ between control and arginine-supplemented rats. In contrast, both hArg concentrations and AGAT activity in the kidney were enhanced in response to L-arginine supplementation (Table 8). Thus, it is likely that the kidneys, but not the liver or the gut, contribute to an increase in the circulating level of hArg in arginine-supplemented rats. Additionally, our results suggest tissue-specific regulation of AGAT expression by L-arginine.

In conclusion, supplementing 1.8 and 3.6 g L-arginine/kg BW/day for 91 days did not have any adverse effects on the measured physiological variables or general health status of male or female rats. Our results also indicate a promising effect of L-arginine on improving lean tissue mass and metabolic profiles in the plasma, while beneficially reducing plasma concentrations of ammonia, free fatty acids, and triglycerides, as well as white adipose tissue in the body. L-Arginine supplementation enhanced AGAT activity in the kidneys but not in the liver or the small intestine, suggesting a tissue-specific difference in AGAT expression to regulate arginine metabolism in the body. Collectively, the findings from the present study are helpful for predicting a safe upper limit for oral administration of L-arginine to healthy adults and in guiding clinical studies to determine long-term safety of L-arginine supplementation in humans.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The use of animals for this research was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

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