Oral arginine improves linear growth of long bones and the neuroendocrine mechanism

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Abstract: Objective To investigate the effect of oral administration of arginine on linear growth of long bones in male pubertal rats and the underlying mechanisms, focusing on expression of genes related to the hypothalamus-pituitary growth axis and the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway. **Methods** Rats were randomly divided into control and intervention groups. In the intervention group, arginine was solved in water (0.045 g *L*-arginine was mixed with 1 mL water) and administered in rats (10 mL/kg) through gastric perfusion once per day, for totally 28 d. Rats in the control group received normal saline treatment. Bone histomorphometry analysis was used to measure growth plate width and mineral apposition rate of the tibia, as well as trabecular bone volume fraction, osteoblast surface and osteoclast surface of the femur. Serum growth hormone (GH) concentration was determined by radioimmunoassay. Real-time PCR was used to measure the expression of neuronal nitric oxide synthase (*nNOS*), soluble guanylyl cyclases (*sGCα1* and *sGCβ1*), growth hormone-releasing hormone (*Ghrh*) and somatostatin (*SS*) in hypothalamus, as well as *Gh* in pituitary. Western blot was used to detect the protein levels of nNOS, sGCα1 and sGCβ1 in hypothalamus. **Results** After treatment with arginine, the growth plate width of tibia and osteoblast surface of femur were increased ($P < 0.05$), and serum GH concentration was elevated ($P < 0.05$). Besides, mRNA and protein levels of nNOS and sGCα1 ($P < 0.05$), as well as the expression of *Gh* mRNA ($P < 0.01$), were significantly up-regulated, while the expression of *SS* mRNA was down-regulated ($P < 0.05$). **Conclusion** Oral administration of arginine could improve linear growth of long bones by regulating mRNA expression of *SS* and *Gh* and inducing GH secretion, possibly via nNOS-NO-sGC-cGMP signal transduction pathway.

Keywords: arginine; nitric oxide synthase; nitric oxide; growth hormone; bone histomorphometry

1 Introduction

Puberty is a specific period of growth and development of children, characterized by growth spurt, resulting from the co-regulation of hypothalamus-pituitary growth

axis and hypothalamus-pituitary-sexual gland axis. It has been recognized that growth hormone (GH) plays an important role in the linear growth of long bones from childhood to adolescence. GH is regulated by hypothalamic growth hormone releasing hormone (GHRH) and somatostatin (SS), which stimulates and inhibits GH secretion, respectively^[1-3].

Clinically, drug interventions are widely used to promote the height growth of children with short stature. Sub-

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cutaneous injection of recombinant human GH can accelerate linear growth of long bones and improve final height. *L*-arginine is a potent stimulator of GH secretion^[4-8], but the mechanism has not been fully clarified. Growing evidence supports that nitric oxide (NO) is a major regulatory signal involved in the hypothalamus-pituitary effects of *L*-arginine^[9]. The present study was aimed to observe the effect of oral arginine on linear growth of long bones and to explore the action mechanism, focusing on the role of neuronal nitric oxide synthase (nNOS)-NO-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway in promoting the function of hypothalamus-pituitary growth axis.

2 Materials and methods

2.1 Animals Fifty-four male Sprague-Dawley rats (5-week-old, in puberty) were provided by Experimental Animal Department of Fudan University of Shanghai. They were housed at standard temperature (23±1) °C under a 12:12 h light/dark cycle, with *ad libitum* access to food and water, in the Animal Center of Fudan University of China. The rats were randomly divided into control and intervention groups ($n = 27$ in each group). In the control group, rats received gastric perfusion of normal saline (10 mL/kg) once per day, for totally 28 d. In the intervention group, arginine (Nanjing Pharmaceutical Factory Co., LTD., China) was solved in water (0.045 g *L*-arginine was mixed with 1 mL water) and administered in rats (10 mL/kg) through gastric perfusion, once per day for totally 28 d. The dosage and feeding time of arginine were determined based on the previous studies in humans and rats^[10-12]. In each group, 6 rats were randomly selected for histomorphometric measurement, and they were intraperitoneally injected with tetracycline (30 mg/kg; Sigma, St. Louis, MO, USA) and calcein (15 mg/kg; Sigma, St. Louis, MO, USA) on days 16 and 26, respectively.

2.2 Sample preparation All animals were anesthetized on day 28 with 11% Urethane (1 mL per 100 g body weight) from abdominal cavity. Femurs and tibias were harvested from 12 rats for histomorphometry. Left tibias were preserved in 70% ethanol at 4 °C. Right femurs and

tibias were fixed in periodate-lysine-paraformaldehyde (PLP) fixative at 4 °C for 3 d. Blood, hypothalamus and pituitary samples were collected from the remaining 42 rats. Blood samples were immediately centrifuged at 2 000 rpm for 20 min (4 °C), and serum were stored at -20 °C until use. Brain was placed on their dorsal surface and dissected as described previously^[13]. In brief, vertical coronal cuts were made through the body of the optic chiasm (tangential to the olfactory tubercles) and immediately posterior to the infundibular stem. The resulting brain slice was turned on its posterior surface. The hypothalamus was removed in a block by cutting along perihypothalamic sulci and then horizontally immediately above the anterior commissure. Removal of hypothalamus and pituitary samples was operated by Wen-Jiang ZHOU from Experimental Animal Department of Fudan University, and samples were kept in liquid nitrogen.

2.3 Bone histomorphometric measurements Left tibias were embedded in plastic. Serial sections were prepared using a non-decalcified microtome. Mineral apposition rate ($\mu\text{m}/\text{d}$) was determined by dividing the mean of the width of the double labels by the interlabel time (10 d). Right femurs and tibias were decalcified with EDTA-G at 4 °C for 4 weeks. The specimens were then dehydrated in an ascending ethanol series and embedded in paraffin. Serial sections (5 μm in thickness) were prepared using a microtome. After hematoxylin-eosin (HE), alkaline phosphatase (ALP), or tartrate-resistant acid phosphatase (TRAP) staining, growth plate width of the tibia and trabecular bone volume fraction (percentage of total marrow cavity that is occupied by cancellous bone; BV/TV, %), osteoblast surface and osteoclast surface of the femur were calculated. The parameters measured for bone structure and mass were the growth plate width (μm) and the trabecular BV/TV (%). The parameter obtained for bone formation was the osteoblast surface per bone surface (Ob.S/BS, %). The parameter measured for bone resorption was the osteoclast surface per bone surface (Oc.S/BS, %). All digital images of histological sections were captured using a microscope (Nikon E600, Japan) and histomorphometric measurements were made using Image Pro Plus (Media Cybernet-

ics, Washington, USA).

2.4 Radioimmunoassay The concentration of serum GH was measured in a volume of 25–50 μ L using Rat Growth Hormone RIA Kit (RGH-45HK, LINCO Research, MO, USA) following the manufacturer's protocol.

2.5 Real-time PCR analysis Total RNA was extracted from hypothalamus and pituitary tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (2 μ g) was reverse-transcribed into cDNA by using M-MLV Reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) and 0.5 μ mol/L of primers specific for rat *nNOS*, *sGC α 1*, *sGC β 1*, *Ghrh*, *SS*, *Gh* and β -*actin* using the Mastercycler ep realplex (Eppendorf, Germany). The sequences and annealing temperatures (T_A) of the primers were as follows: *nNOS* forward 5'-gacaacgttctgtgtct-3', reverse 5'-tccagtgtgctttcaggtg-3' (T_A = 60 °C); *sGC α 1* forward 5'-ctttgtgtcaccgccagat-3', reverse 5'-cagaacatgcggctcacta-3' (T_A = 56 °C); *sGC β 1* forward 5'-acggtcccgaggtgtggaag-3', reverse 5'-tccaggacacgcaagatggt-3' (T_A = 60 °C); *Ghrh* forward 5'-actctgggtgttcttgtgc-3', reverse 5'-agcttcaccttgggaatcc-3' (T_A = 59 °C); *SS* forward 5'-acccagactccgtcagttt-3', reverse 5'-gaagtcttgcagccagctt-3' (T_A = 60 °C); *Gh* forward 5'-gcagagaactgacatggaattg-3', reverse 5'-ttttgagcagagcgtcatc-3' (T_A = 60 °C); β -*actin* forward 5'-tgacaggatgcagaaggagat-3', reverse 5'-gatagagccaccaatccacaca-3' (T_A = 60 °C). The PCR products were separated in 2% agarose and visualized under UV light. The resulting images were captured by Gel DocTM EI imager (Bio-Rad, CA, USA). The cDNA content was normalized using the copies of target gene divided by the gene copies of β -*actin*, and the gene expression level was the logarithm of normalized cDNA content.

2.6 Western blot Hypothalamus tissue from rat was homogenized on ice in an appropriate volume of homogenization buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 1 mmol/L EDTA; all from Beyotime, China), and centrifuged at 12 000 rpm at 4 °C for 30 min.

The supernatant was collected and protein concentration was determined by using a BCA kit (Beyotime, China). After being heated at 95 °C for 10 min, samples (60 μ g) were separated by SDS-PAGE (8% for nNOS, 10% for sGC α 1 and sGC β 1). Proteins were then transferred to nitrocellulose membranes and blocked in 5% non-fat milk. After being washed for 3 times, membranes were incubated with rabbit anti-nNOS monoclonal antibody (Abcam, United Kingdom; 1:250), rabbit anti-sGC α 1 antibody (Sigma, St. Louis, MO, USA; 1:2 500), or rabbit anti-sGC β 1 antibody (Cayman Chemicals, Ann Arbor, MI, USA; 1:200). After being washed, membranes were incubated with secondary antibody (1:4 000). Proteins were visualized with the SuperSignal West Pico Chemiluminescent Substrate Trial Kit (Thermo, Rockford, IL, USA). HRP-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from KangChen Biotech Inc., China. Results were captured using the LAS-4000 mini (Fujifilm, Japan) and the computerized image analysis system (Muti Gauge V3.2, Fujifilm, Japan).

2.7 Statistical analysis Data were expressed as mean $\pm$ SEM and analyzed by *t*-test using SPSS 15.0 software. $P < 0.05$ was indicated as statistically significant.

3 Result

3.1 Effect of arginine on bone metabolism After intervention with arginine, the growth plate width of tibia and osteoblast surface of femur were increased ($P = 0.048$, $P = 0.030$, respectively). The mineral apposition rate of the tibia ($P = 0.109$), as well as trabecular bone volume fraction ($P = 0.253$) and osteoclast surface ($P = 0.188$) of the femur, was also up-regulated, but with no statistical significance (Tables 1, 2).

Table 1. Comparison of growth plate width (GP) and mineral apposition rate (MAR) of the tibia between the 2 groups (mean $\pm$ SEM)

Groups	<i>n</i>	GP (μ m)	MAR (μ m/d)
Control	6	218.36 $\pm$ 5.25	1.23 $\pm$ 0.14
Intervention	6	243.86 $\pm$ 9.09*	1.82 $\pm$ 0.28

* $P < 0.05$ vs the control.

Table 2. Comparison of trabecular bone volume fraction (BV/TV, %), osteoblast surface (Ob.S/BS, %) and osteoclast surface (Oc.S/BS, %) of the femur between the 2 groups (mean±SEM)

Groups	<i>n</i>	BV/TV (%)	Ob.S/BS (%)	Oc.S/BS (%)
Control	6	18.01±3.18	34.26±3.23	32.22±3.61
Intervention	6	22.62±2.82	45.74±3.18*	35.22±2.66

**P* < 0.05 vs the control. BV/TV: percent of total marrow cavity that is occupied by cancellous bone. Ob.S/BS: osteoblast surface per bone surface. Oc.S/BS: osteoclast surface per bone surface.

Fig. 1 Comparison of serum growth hormone (GH) concentration between the 2 groups of rats. Data were expressed as mean±SEM (*n* = 21) and analyzed by *t*-test. **P* < 0.05 vs the control.

3.2 Effect of arginine on serum GH concentration As shown in Fig. 1, the concentration of serum GH was significantly increased following oral administration of arginine, compared with the control group (*P* = 0.015).

3.3 Effects of arginine on gene expression of *nNOS*, *sGCα1*, *sGCβ1*, *Ghrh* and *SS* in hypothalamus and *Gh* in pituitary The PCR products were shown in Fig. 2. Expression of *nNOS*, *sGCα1*, and *Gh* was up-regulated (*P* value: 0.022, 0.043, 0.001, respectively), and gene expression of *SS* was down-regulated (*P* = 0.045), following oral administration of arginine. Besides, the expression levels of *sGCβ1* and *Ghrh* were increased in the intervention group, but with no statistical significance (*P* = 0.507, *P* = 0.124, respectively) (Table 3).

3.4 Effects of arginine on protein expression of hypothalamus *nNOS*, *sGCα1* and *sGCβ1* As shown in Fig. 3,

Fig. 2 PCR products of *nNOS* (179 bp), *sGCα1* (201 bp), *sGCβ1* (224 bp), *Ghrh* (294 bp) and *SS* (242 bp) in rat hypothalamus and *Gh* (269 bp) in pituitary. Lane 1: control group; Lane 2: intervention group. *β-Actin* served as the internal control (106 bp).**Table 3.** Comparison of gene expression of *nNOS*, *sGCα1*, *sGCβ1*, *Ghrh* and *SS* in hypothalamus and *Gh* in pituitary between the 2 groups (mean±SEM)

Genes	Control (<i>n</i> = 15)	Intervention (<i>n</i> = 15)
Hypothalamus		
<i>nNOS</i>	2.45±0.05	2.66±0.06*
<i>sGCα1</i>	2.64±0.05	2.79±0.07*
<i>sGCβ1</i>	1.43±0.06	1.48±0.05
<i>Ghrh</i>	1.10±0.07	1.29±0.08
<i>SS</i>	1.39±0.08	1.11±0.09*
Pituitary		
<i>Gh</i>	2.06±0.11	2.73±0.06**

P* < 0.05, *P* < 0.01, vs the control. Data were the logarithm of gene copies normalized to *β-actin*.

the protein levels of *nNOS* and *sGCα1* were significantly up-regulated (*P* = 0.031, *P* = 0.045, respectively) following oral administration of arginine. However, the protein level of *sGCβ1* showed no significant alteration (*P* = 0.653) after arginine administration.

4 Discussion

GH is an important modulator of longitudinal bone growth and appears as a central player of the hypothalamus-pituitary growth axis^[14]. After being secreted by the pituitary gland, GH exerts its effect on epiphyseal growth plate of the long bone either directly or through insulin-like growth factor-1 (IGF-1), which activates chondro-

Fig. 3 A: Western blot results of proteins nNOS (161 kDa), sGCα1 (80 kDa) and sGCβ1 (70 kDa) in rat hypothalamus. GAPDH served as the internal control (37 kDa). Lane 1, the control group. Lane 2, the intervention group. B: OD values of the proteins were normalized to that of GAPDH. Results were expressed as mean±SEM ($n = 6$). Lane 1, the control group. Lane 2, the intervention group. * $P < 0.05$ vs the control.

cyte proliferation and differentiation in the growth plate, thereby achieving longitudinal growth. Our data indicated that oral arginine increased the growth plate width of tibia and osteoblast surface of femur by improving *Gh* mRNA expression and serum GH concentration, resulting in the linear growth of long bones.

L-arginine increases GH release in pituitary when given orally or intravenously^[11,15], the mechanism of which is believed to be through inhibition of endogenous SS release^[2,5]. Similarly, here we showed that oral arginine mainly stimulated GH release through inhibiting the expression of hypothalamic *SS* mRNA. In addition, it has been reported that NO, the metabolite of arginine, is involved in the control of GH secretion^[16].

nNOS has been revealed to be present in hypothalamic nuclei and pituitary cells^[17,18], where *L*-arginine is metabolized to NO and *L*-citrulline. NO has been shown to be involved in the control of secretion of many hormones along the hypothalamic-pituitary axis including the somatotrope axis^[19-23]. An important mechanism underlying the action of NO involves activation of sGC, stimulating the

formation of cGMP, a process also occurring in the neuroendocrine system^[24,25]. Luque *et al.*^[26] have found that ghrelin, a peptide isolated from the stomach and presenting strong GH-releasing activity, induces GH secretion through a mechanism involving NOS-NO-sGC-cGMP signaling pathway. Our results imply that *L*-arginine induces hypothalamic nNOS expression and increases NO release in the central nervous system. In addition, *L*-arginine inhibits *SS* mRNA expression and stimulates GH secretion, possibly through nNOS-NO-sGC-cGMP signaling pathway.

However, animals and humans subjected to arginine treatment present reduced arterial blood pressure values, an effect supposed to occur in response to the dilating effect of NO generated from *L*-arginine^[12,27,28]. However, arginine administration at an appropriate dose is safe. For example, neonatal pigs, growing-finishing pigs, pregnant pigs, and adult rats tolerate large amounts of chronic supplemental arginine (at least 0.62, 0.32, 0.21, and 2.14 g/kg body weight per day, respectively) administered via enteral diets without any adverse effects^[29]. Additionally, oral *L*-arginine (9 g per day for adults) has no adverse effects in

humans^[30].

In conclusion, oral arginine could significantly promote the linear growth of long bones by enhancing bone formation versus bone resorption. The mechanism may be that *L*-arginine, via the nNOS-NO-sGC-cGMP signaling pathway, promotes the function of hypothalamus-pituitary growth axis, resulting in enhanced synthesis and secretion of GH. Thus, arginine may represent a nutritional strategy potentially advantageous for prevention and treatment of some GH disorders^[12]. This study analyzed the mechanism underlying oral arginine-induced improvement in linear growth of long bones from the perspective of neuroendocrine, and provided a theoretical basis for clinical treatment.

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口服精氨酸促进长骨线性生长的作用及神经内分泌调节机制

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摘要: **目的** 观察口服精氨酸对雄性青春期大鼠长骨线性生长的影响, 并从下丘脑-垂体生长轴及一氧化氮-环磷酸鸟苷通路相关的基因表达的角度探讨其机制。 **方法** 将青春期大鼠分为对照组和干预组, 干预组大鼠每天灌胃 10 mL/kg 精氨酸水溶液 (0.045 g/mL), 共给药 28 天。对照组大鼠每天给予同剂量的生理盐水。采用骨形态计量学方法分别测定胫骨生长板宽度和矿化沉积率, 以及股骨骨小梁体积百分数、成骨细胞面积及破骨细胞面积。用放射免疫法测定血清中生长激素的浓度。实时定量聚合酶链式反应检测下丘脑神经型一氧化氮合酶(*nNOS*)、可溶性鸟苷环化酶(*sGCα1/sGCβ1*)、生长激素释放激素(*Ghrh*)、生长抑素(*SS*)及垂体*Gh*基因表达水平。Western blot 法测定下丘脑*nNOS*、*sGCα1*及*sGCβ1*的蛋白表达水平。 **结果** 口服精氨酸后, 青春期大鼠胫骨生长板宽度及股骨成骨细胞面积明显增加($P < 0.05$), 血清生长激素浓度显著提高($P < 0.05$)。此外, 下丘脑*nNOS*和*sGCα1*的基因及蛋白表达水平均显著上调($P < 0.05$), 而*SS*基因表达水平显著下调($P < 0.05$)。垂体*Gh*基因表达显著上调($P < 0.01$)。 **结论** 口服精氨酸可能通过调控*nNOS*-NO-*sGC*-cGMP通路使得下丘脑*SS*的基因表达下调、垂体*Gh*基因表达上调、垂体生长激素分泌增多, 从而显著促进长骨的线性生长。

关键词: 精氨酸; 一氧化氮合酶; 一氧化氮; 生长激素; 骨形态计量学