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Liquorice flavonoid oil increased skeletal muscle thickness as assessed by ultrasound in training football athletes

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Abstract

Liquorice flavonoid oil (LFO) consisting of liquorice hydrophobic polyphenols in medium-chain triglycerides (MCT) is a new functional food ingredient effective for preventing metabolic syndrome. As it has been recently shown that LFO significantly increased skeletal muscle mass in mice, we hypothesized that it would also increase skeletal muscle mass in humans. Therefore, we carried out a randomized, double-blind, placebo-controlled study with male football athletes who ingested 300 mg per day of LFO concentrate solution for 8 weeks during the course of training at Kindai University, Osaka. Ultrasound imaging analysis revealed that the muscle thickness of the anterior thighs and anterior brachial regions in the LFO group were both significantly increased by 2.5% at week 8 in comparison to baseline, but not in the placebo group. In addition, although abdominal muscle thickness significantly increased in both the LFO and the placebo groups at week 8 in comparison to baseline, the increase in the LFO group was 1.8 times greater than that in the placebo group (p<0.05). Interestingly, stratified analysis by two team positions revealed that the offensive backs group, who require speed, gained more anterior thigh muscle, while the defensive line group, who require pushing force, gained more anterior branchial muscle with repeated intake of LFO. These results correlate well with the position-specific training of the two groups. This study indicates that LFO can contribute as a dietary supplement ingredient to increase or maintain skeletal muscle mass in humans in combination with exercise. This is the first report showing that LFO, which consists of liquorice polyphenols, increases muscle mass in humans.

Introduction

Increase in and maintenance of skeletal muscle mass in humans is important to improve athletic performance and also to prevent sarcopenia, which is an age-related decrease in muscle mass [1]. In addition to appropriate exercise, active uptake of nutrients for muscle growth has been proven to be effective for increasing skeletal muscle mass.
Indeed, some proteins and amino acids are known to promote increased skeletal muscle mass [2].

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In recent years, non-nutritive functional components obtained from plant-derived foods, such as polyphenols including grape seed proanthocyanidins and tea catechins, have received a great deal of attention worldwide because they exert a variety of preventative effects on diseases such as coronary heart disease [3], diabetes [4] and cancer [5]. Furthermore, it has been recently shown that the polyphenol resveratrol increased muscle mass in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy [6].

Liquorice flavonoid oil (LFO), which is derived from liquorice (*Glycyrrhiza glabra* L.) is a functional food ingredient consisting of liquorice hydrophobic polyphenols in medium-chain triglycerides (MCT). We initially demonstrated that LFO ameliorated abdominal obesity and diabetes in metabolic syndrome mouse models [7]. Since then, a number of studies have confirmed the preclinical safety of LFO in vitro and in vivo, including a 90-day subchronic toxicity study in rats [8], genotoxicity studies [9], a medium-term liver bioassay for carcinogenesis [10] and a bioavailability study [11]. Subsequently, human safety research that included single and repeated dose studies, also confirmed the ingredient's safety with no adverse events (AEs) [12]. Studies in human subjects have shown that LFO was effective in reducing total body fat and visceral fat in Japanese overweight subjects with a body mass index (BMI) of up to 30 kg/m^2 and in American obese subjects with a BMI of up to 40 kg/m^2 [13–15]. Investigation of the mechanism by which LFO suppresses fat accumulation using DNA microarray analysis and subsequent enzymatic analysis with mice and rats fed a high-fat diet and LFO revealed that LFO up-regulated genes for beta-oxidation and down-regulated those for fatty acid synthesis [16, 17].

Subsequently, it has been demonstrated that single and repeated intake of LFO enhanced fat metabolism in humans [18].

Recently, we evaluated changes in abdominal fat and femoral muscle weights in obese and diabetic KK-Ay mice after 1 month of ad libitum feeding with a diet containing 1% LFO concentrate solution or gavage administration of 1000 mg/kg LFO concentrate solution. The results indicated that the weight of abdominal fat significantly decreased, while femoral muscle weight significantly increased (unpublished data). Therefore, we hypothesized that intake of LFO would be able to increase skeletal muscle mass in humans. If effective, LFO may be useful in human nutrition both for the prevention of sarcopenia, which is defined as age-related muscle wasting [1], and for improving physical performance in athletes by increasing muscle mass. The aim of this study was to evaluate the efficacy of LFO in increasing muscle mass in humans. Therefore, we conducted a randomized, double-blind, placebo-controlled study in healthy male football athletes during the course of training. In this study, we used B-mode ultrasound to assess the muscle mass of the subjects because of the high correlation between values measured by this method and those measured by DXA [19], CT [20] and MRI [21, 22], which are the gold standards. In addition, this method is safe for all populations, portable, and capable of taking the measurements of large numbers of training athletes in the field.

Methods and materials

Subjects and data sets analyzed

The subjects eligible for the study consisted of 31 healthy male football athletes with an average age of 20.1±0.8 years and average body weight of 91.6±13.7 kg. The subjects were members of the Kindai University football team, which belongs to Division 1 of the Kansai American Football League. Forty subjects initially started the study, but nine (five from the LFO group, and four from the placebo group) dropped out: six subjects dropped out due to injury during training, one due to illness, and two due to excessive food intake as shown by their diet diaries. Thus, 31 subjects were included in the efficacy analysis, of whom 15 were in the LFO group and 16 were in the placebo group.

Test food product

The LFO test material was prepared as follows. An ethanol extract of liquorice (*G. glabra* L.) root was obtained. The ethanolic layer was then mixed with MCT and the concentration of glabridin, the major component of the solution, was adjusted to 3% (w/w). This solution was called the 'LFO concentrate solution', which has the brand name Kaneka Glavonoid®. Soft gelatin capsules containing 300

mg LFO concentrate solution per capsule together with MCT and beeswax were used for the LFO group, while capsules containing only MCT and beeswax were used for the placebo group.

Study design

This was a randomized, double-blind, placebo-controlled study performed at the division of Food Science of Nutrition in the Faculty of Agriculture, Kindai University, Osaka. The protocol was approved by the ethics committee of the Faculty of Agriculture of Kindai University in accordance with the concepts of the Helsinki declaration. Written informed consent was obtained from all subjects after a full explanation of the study, including its aims, procedures and ethical care, was provided. Written informed consent was obtained from the parents of minor subjects under the age of 20. Each subject was randomly allocated to either the LFO or placebo group, and then received the test food product. Fifteen subjects received LFO, and 16 subjects received placebo capsules in a plastic bottle. At baseline, the thickness of muscle and subcutaneous fat was measured using digital ultrasonography, and athletic performance was evaluated. The results showed that there was no significant difference between the measurements in the LFO and placebo groups. The subjects then ingested one capsule containing 300 mg of LFO or placebo daily for 8 weeks. The athletes returned at the end of week 8 and the same measurements as at baseline were again performed.

Measurement of muscle and subcutaneous fat thickness

Muscle and subcutaneous fat thickness was determined at a club centre at Kindai University using a personal digital ultrasound imaging device (Global Health, Kanazawa) operated by the same experienced operator at baseline and at the end of week 8 in accordance with the manufacturer's instructions. The measurement sites were the anterior region of the thigh, the abdominal area, and the anterior brachial region.

Measurement of athletic performance

Athletic performance was assessed by the box jump and 200-yard shuttle run at the club centre at Kindai University.

Statistical analysis

Data are presented as means±standard deviation (SD). For the efficacy analyses, a paired t-test (two-sided) was used to detect significant changes due to ingestion within each group. The significance of the difference between the LFO and placebo group was determined using the F test. If the data were found to be homogeneous, they were analyzed with Student's t-test (two-sided); otherwise, they were tested with the Welch test (two-sided). A p value of less than 0.05 was considered significant.

Results

Muscle thickness of the anterior thigh, abdominal and anterior brachial regions

No subject complained of physical discomfort caused by the repeated ingestion of LFO throughout the study. The absolute values of muscle and subcutaneous fat at baseline and at week 8 are shown in Table 1, and the changes in thickness of muscle and subcutaneous fat at week 8 compared to baseline are shown in Table 2.

		LFO				Placebo			
			Baseline	Week 8			Baseline	Week 8	
		N	Mean ± SD	Mean ± SD		N	Mean ± SD	Mean ± SD	
Thickness of muscle									
Anterior region of thigh	(mm)	15	65.8 ± 7.3	67.5 ± 5.8	*	16	63.0 ± 6.7	63.5 ± 6.7	
Abdominal area	(mm)	14	16.5 ± 2.3	19.0 ± 2.6	***	16	17.1 ± 1.7	18.5 ± 1.9	**
Anterior brachial region	(mm)	15	34.7 ± 2.9	35.6 ± 3.1	*	16	35.9 ± 3.6	36.3 ± 3.3	
Thickness of subcutaneous fat									
Anterior region of thigh	(mm)	15	7.5 ± 1.9	6.8 ± 1.9	**	16	7.9 ± 2.6	7.4 ± 2.3	*
Abdominal area	(mm)	14	20.8 ± 8.7	23.4 ± 11.2		16	15.7 ± 10.3	16.4 ± 9.9	
Anterior brachial region	(mm)	15	4.0 ± 1.5	4.0 ± 1.5		16	3.5 ± 1.1	3.3 ± 1.2	

*p<0.05, **p<0.01, ***p<0.001; significant difference compared to baseline by paired t-test
LFO liquorice flavonoid oil

Table 1 - Absolute values of thickness of muscle and subcutaneous fat at baseline and at week 8

		LFO		Placebo	
		N	Mean ± SD	N	Mean ± SD
Thickness of muscle					
Anterior region of thigh	(mm)	15	1.7 ± 2.4	16	0.5 ± 2.4
Abdominal area	(mm)	14	2.5 ± 1.2	16	1.4 ± 1.5 #
Anterior brachial region	(mm)	15	0.9 ± 1.6	16	0.4 ± 1.8
Thickness of subcutaneous fat					
Anterior region of thigh	(mm)	15	-0.7 ± 0.7	16	-0.5 ± 0.8
Abdominal area	(mm)	14	2.6 ± 5.7	16	0.7 ± 3.6
Anterior brachial region	(mm)	15	0.0 ± 0.6	16	-0.1 ± 0.3

#$p<0.05$; significant difference between LFO and placebo groups by Student's *t*-test
LFO liquorice flavonoid oil

Table 2 - Changes in thickness of muscle and subcutaneous fat at week 8 compared to baseline

The absolute value of anterior thigh muscle thickness was significantly increased by 2.5% at week 8 compared to baseline ($p<0.05$) in the LFO group but not in the placebo group.
The absolute value of abdominal muscle thickness in both the LFO and placebo groups was significantly increased at week 8 compared to baseline ($p<0.001$ in the LFO group, $p<0.01$ in the placebo group). However, the increase in abdominal muscle thickness in the LFO group was 1.8 times greater than that in the placebo group ($p<0.05$). The absolute value for anterior brachial muscle thickness was significantly increased by 2.5% at week 8 compared to baseline ($p<0.05$) in the LFO group but not in the placebo group.

Subcutaneous fat thickness in the anterior thigh, abdominal and anterior brachial regions

The absolute values for fat thickness at baseline and at week 8 are shown in Table 1, while the changes in fat thickness at week 8 compared to baseline are shown in Table 2. The absolute value for anterior thigh subcutaneous fat thickness in the LFO group was significantly decreased at week 8 compared to baseline ($p<0.01$). That in the placebo group was also significantly decreased at week 8 compared to baseline ($p<0.05$), but there was no significant difference between the groups. The absolute value for abdominal and anterior brachial subcutaneous fat thickness in the LFO and placebo groups showed no significant change at week 8 compared to baseline, and there was no significant difference between the groups.

Performance measurement

The box jump and the 200-yard shuttle run were conducted to evaluate instantaneous power and to evaluate agility and muscle endurance, respectively. The absolute values of the box jump and 200-yard shuttle run at baseline and at week 8, and the change in those values at week 8 compared to baseline are shown in Table 3. The results of the box jump measurement indicate the number of times subjects in both groups showed no change at week 8 compared to baseline. The results of the 200-yard shuttle for both groups show significant decreases in time taken to complete the run at week 8 compared to baseline ($p<0.001$ in both groups).

Stratified analysis by position (line and backs)

In American football, different physiques, skills and, consequently, training are required for each position (offensive line, tight end, quarterback, running back, wide receiver, defensive line, linebacker, defensive back and kicker). For example, higher weight and more arm power are required in the line group, while greater speed, leg power and agility are required in the backs group. The offensive line group in our study focused on training using a piece of equipment called a blocking sled (used to develop arm power), while the defensive backs group focused on sprint training to improve leg power. There-

		LFO				Placebo			
			Baseline	Week 8			Baseline	Week 8	
		N	Mean ± SD	Mean ± SD		N	Mean ± SD	Mean ± SD	
Absolute value									
Box jump	(times)	15	13.8 ± 1.0	13.6 ± 1.3		14	13.7 ± 1.8	13.7 ± 1.5	
200-Yard shuttle run	(seconds)	15	32.9 ± 2.3	30.7 ± 2.1	***	14	34.2 ± 5.2	31.6 ± 3.3	***
Change									
Box jump	(times)	15	N/A	-0.2 ± 1.2		14	N/A	0.0 ± 1.6	
200-Yard shuttle run	(seconds)	15	N/A	-2.2 ± 1.8		14	N/A	-2.6 ± 2.2	

***$p<0.001$; significant difference compared to baseline by paired *t*-test
LFO liquorice flavonoid oil

Table 3 - Absolute values and changes in the box jump and 200-yard shuttle run at baseline and at week 8

fore, in order investigate the difference in the effect of LFO on skeletal muscle and body fat mass between the offensive line group and the defensive backs group, we performed stratified analysis in the LFO and placebo groups for the two positions. There were eight line group and seven backs group subjects in the LFO group, and six line group and 10 backs group subjects in the placebo group. Table 4 shows the results of the stratified analysis of absolute values of muscle and subcutaneous fat thickness, box jump and 200-yard shuttle run at baseline and at week 8 for the line and backs positions.

As shown Table 4, in the line group, the absolute value for anterior brachial muscle thickness in the LFO group was significantly increased at week 8 compared to baseline, but not in the placebo group, although there was no significant difference between the LFO and placebo groups at week 8. On the other hand, in the backs group, there was no significant difference in the absolute value for anterior brachial muscle thickness at week 8 compared to baseline in both the LFO and placebo groups. In the backs group, the absolute value for anterior thigh muscle thickness in the LFO group was significantly increased at week 8 compared to baseline, but not in the placebo group, although there was no significant difference between the LFO and placebo groups at week 8. On the other hand, in the line group, there was no significant difference in anterior thigh muscle thickness at week 8 compared to baseline in both the LFO and placebo groups. The absolute values for abdominal muscle thickness in the LFO and placebo groups at week 8 compared to baseline were significantly increased in both the line and the backs groups. The absolute values for anterior thigh subcutaneous fat thickness of the LFO and placebo groups in the line group, and those of the LFO group in the backs group were significantly decreased at week 8 compared to baseline. However, a significant difference was not observed between the LFO and placebo groups in either the line or the backs groups. Regarding performance measurement, as shown in Table 4, the times of the 200-yard shuttle run in the LFO and placebo groups were significantly decreased at week 8 compared to baseline in the line and the backs groups.

Discussion

In order to determine if LFO is effective at increasing muscle mass in humans, we performed a randomized, double-blind, placebo-controlled study of male American football athletes who ingested 300 mg LFO concentrate solution once daily for 8 weeks during the course of training at Kindai University. B-mode ultrasonography was used to

		Line								Backs							
		LFO				Placebo				LFO				Placebo			
			Baseline	Week 8			Baseline	Week 8			Baseline	Week 8			Baseline	Week 8	
		N	Mean ± SD	Mean ± SD		N	Mean ± SD	Mean ± SD		N	Mean ± SD	Mean ± SD		N	Mean ± SD	Mean ± SD	
Thickness of muscle																	
Anterior region of thigh	(mm)	8	68.9 ± 5.7	69.5 ± 4.6		6	67.9 ± 6.7	69.4 ± 5.7		7	62.3 ± 7.7	65.2 ± 6.5	**	10	60.1 ± 5.0	60.0 ± 4.6	
Abdominal area	(mm)	8	17.7 ± 2.3	20.3 ± 2.6	***	6	16.8 ± 1.3	18.5 ± 1.3	*	6	15.0 ± 1.2	17.3 ± 1.5	**	10	17.3 ± 2.0	18.6 ± 2.2	*#
Anterior brachial region	(mm)	8	35.9 ± 2.5	37.2 ± 2.0	**	6	37.2 ± 4.1	38.3 ± 3.1		7	32.3 ± 3.0	33.7 ± 3.1		10	35.1 ± 3.3	35.1 ± 2.9	
Thickness of subcutaneous fat																	
Anterior region of thigh	(mm)	8	8.3 ± 1.9	7.7 ± 1.9	**	6	10.1 ± 1.6	9.1 ± 0.9	*	7	6.6 ± 1.5	5.8 ± 1.6	*	10	6.6 ± 2.2	6.4 ± 2.3	
Abdominal area	(mm)	8	24.1 ± 8.8	27.7 ± 11.2		6	25.4 ± 8.8	26.0 ± 7.9		6	16.4 ± 7.0	17.8 ± 9.2		10	9.8 ± 5.6	10.6 ± 5.6	
Anterior brachial region	(mm)	8	4.9 ± 1.5	4.9 ± 1.4		6	4.6 ± 1.1	4.6 ± 1.0		7	3.0 ± 0.6	2.9 ± 0.6		10	2.8 ± 0.4	2.6 ± 0.5	**
Athletic performance																	
Box jump	(times)	8	13.3 ± 0.6	12.9 ± 0.8		6	12.7 ± 1.9	13.0 ± 1.3		7	14.5 ± 0.9	14.5 ± 1.2		8	14.5 ± 1.5	14.3 ± 1.5	
200-Yard shuttle run	(seconds)	8	33.8 ± 2.0	31.8 ± 2.1	**	6	37.3 ± 6.8	33.5 ± 4.3	*	7	32.0 ± 2.3	29.6 ± 1.4	*	8	31.9 ± 1.5	30.1 ± 1.1	**

*p<0.05, **p<0.01, ***p<0.001; significant difference compared to baseline by paired t-test
#p<0.05; significant difference between LFO and placebo groups by Student's t-test
LFO liquorice flavonoid oil

Table 4 - Stratified analysis of absolute values of thickness of muscle and subcutaneous fat, box jump and 200-yard shuttle run at baseline and at week 8 by line and backs positions

measure muscle thickness in order to assess the muscle mass of the athletes. Muscle thickness can be used to assess muscle mass because there is a high statistically significant correlation between muscle thickness measured by ultrasound (B-mode ultrasonography) and muscle mass estimated by gold standards such as DXA [19] and MRI [21, 22]. For example, a strong correlation has been shown between site-matched muscle mass (total, arm, trunk body, thigh and lower leg) measured using MRI and muscle thickness×height in 48 Japanese adults (R^2=0.96, p<0.001) [22]. In addition, B-mode ultrasonography is a safe and non-invasive method with no X-ray exposure, and is an economical and simple method suitable for use with large numbers of subjects. Therefore, this method has been widely used for the evaluation of muscle mass in aging [23], in resistance training [24] and in sports athletes [25]. In this study, the thickness of muscle and subcutaneous fat was measured and quantified by the same experienced operator at baseline and at week 8 in accordance with the manufacturer's instructions at the exact same site to minimize measurement error.

The results of this study indicate that the muscle thickness of the anterior thigh and anterior brachial regions was significantly increased at week 8 in comparison to baseline in the LFO group only, while that of the abdomen was significantly increased in both the LFO and placebo groups at week 8 in comparison to baseline, with the increase in the LFO group significantly greater than that in the placebo group. This result indicates that repeated intake of LFO can increase skeletal muscle mass. To our knowledge, this is the first finding that particular polyphenols have this effect in humans.

As LFO was shown to decrease body fat in overweight and obese subjects [13–15], it was expected that LFO would suppress increases in body fat mass in this study. However, although significant decreases in subcutaneous fat thickness in the anterior thigh were observed in the LFO group (p<0.01) and in the placebo group (p<0.05), there were no significant differences between the changes in subcutaneous fat thickness in the anterior thigh, abdomen and anterior brachial regions in the LFO and placebo groups. The reason may be that the subjects in this study, as with American football athletes in general, consumed extra food to maintain and increase their body weight as well as their muscle mass, which might have overwhelmed the ability of LFO to reduce body fat mass. According to the diet diary kept as part of this study, the energy intake of subjects during the study was about 4,000 kcal/day, while that of 18–29-year-old males in the general population is 2,700 kcal/day [26]. In addition, over 30% of total energy intake was derived from fat in our subjects, compared to approximately 25% in 18–29-year-old males in the general population.

In American football, different skills are needed in the different positions, which thus require different types of training and different physiques. The line group needs more weight and more arm power to push, while the backs group needs greater speed and agility. According to the results for the two different positions in this study, although there were significant increases in the thickness of abdominal muscle in the LFO and placebo groups in both positions, significant increases in the thickness of the anterior brachial muscle were observed only in the line group subjects in the LFO group, who developed their arm muscles for quick tackling and blocking. On the other hand, significant increases in anterior thigh thickness were observed only in the backs group subjects in the LFO group, who developed their leg muscles for increased power and agility. These results suggest that repeated ingestion of LFO mainly increased the mass of skeletal muscles subject to frequent use or training.

Regarding the possible mechanism of action of LFO to increase muscle mass, LFO possibly contributes to efficient synthesis of skeletal muscle proteins by promoting the uptake of glucose into skeletal muscle cells. It has been demonstrated that glabridin, a prenylated isoflavone and the major component of LFO, stimulated glucose uptake by activating the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane due to activation of AMP-activated protein kinase (AMPK) in L6 myotubes as well as in the muscle cells of ICR mice with OGTT [27]. In the study, glabridin also induced carbon expenditure with a decrease in intra-myocellular ATP and glycogen in L6 myotubes. These results indicate that LFO may

contribute to increases in skeletal muscle mass by enhancing energy consumption for synthesis of skeletal muscle protein. In addition, LFO may also contribute to increases in skeletal muscle mass by activating SIRT1 (the NAD+-dependent protein deacetylase) in skeletal muscle cells. It has been demonstrated that resveratrol (3,5,4-trihydroxy trans-stilbene), known as an antioxidant [28] and a SIRT1 activator [29], decreased muscle oxidative damage and increased skeletal muscle mass in mdx mice, an animal model of Duchenne muscular dystrophy [6]. As mentioned above [27], glabridin, which is the major component of LFO, activated AMPK in L6 myotubes as well as in the muscle cells of ICR mice. SIRT1 can be activated by AMPK [30], so LFO may contribute to increases in skeletal muscle mass through SIRT1 activation triggered by AMPK activation. Further study is necessary to clarify whether LFO can activate the expression of the SIRT1 gene.

In this study, athletic performance was assessed by the box jump and the 200-yard shuttle run. In the 200-yard shuttle run to evaluate agility and muscle endurance, the LFO and placebo groups showed significantly improved values at week 8 in comparison to baseline, but there was no significant difference between the groups. Therefore, the improvement observed in both groups could be due to the effect of training. Further study is necessary to determine if athletic performance is increased by LFO consumption by considering more appropriate methods for evaluation such as the bench press or squat with a longer ingestion period.

This study had one limitation. Only a limited number of American football athletes were enrolled. Further research is needed to confirm the effects found in populations other than athletes, for example, middle aged or elderly people with a higher prevalence of sarcopenia. In addition, the mechanism of the effect of LFO should be examined in greater depth in in vitro and in vivo studies.

Conclusion

This randomized, double-blind, placebo-controlled study in male football athletes is the first to demonstrate that dietary intake of LFO, which consists of liquorice polyphenols that are non-nutritive functional components derived from plants, can help increase skeletal muscle mass in humans.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, and its later amendments. Informed consent was obtained from all patients for being included in the study.

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