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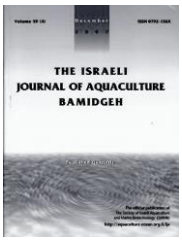

Dietary Supplementation of Glycyrrhetinic Acid (GA) Promoting Lipid Lipolysis from Blunt Snout Bream *Megalobrama amblycephala* by TNF-α and LPL Expression

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Keywords: *Megalobrama amblycephala; Glycyrrhetinic acid; tumor necrosis factor; Lipoprotein lipase; gene expression; fat tissue mass*

Abstract

Glycyrrhetinic acid (GA), which is a main active principal constituent of liquorice, is extensively used as a non-nutrition sweetener or anti-inflammatory in Chinese medicine. This study was carried out to evaluate the effects of different GA levels on growth, biometric parameters, plasma lipid metabolites, Tumor necrosis factor alpha (TNF-α) and Lipoprotein lipase (LPL) gene expression of *Megalobrama amblycephala* (average weight, 68.78 ± 1.13 g). Three diets were formulated to contain various levels of GA (0, 30 and 300 mg/kg). Fish were randomly distributed into 12 cages and fed three times daily for 8 weeks. Dietary GA levels have little significant effect on weight gain and feed conversion ratio. Intraperitoneal fat ratio and viscera/body ratio decreased significantly as dietary GA levels increased, and hepatosomatic index tended to decrease as dietary GA levels increased. Plasma triglycerides, cholesterol, and non-esterified free fatty acid levels also decreased as dietary GA concentrations increased. Adipose tissue and liver TNF-α expression increased significantly with increasing dietary GA levels; whereas, LPL expression showed an opposite trend. Results of this study indicate that dietary supplementation of 300 mg/kg GA had a positive effect in promoting lipid lipolysis in *Megalobrama amblycephala* without having a negative effect on growth through the regulation of TNF-α and LPL expression.

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Introduction

Fat plays a very important role in fish diets; fat-rich feed can improve growth performance of fish (Li et al. 2012). However, high dietary fat leads to excess fat deposition in the liver and other tissues, resulting in fat metabolic disturbance in aquatic species, especially in herbivorous fish (Du et al. 2006; Du et al. 2008; Lu et al. 2013). Previous reports have shown that several nutritional additives improve fat metabolism of mammals and fish, but little information is available concerning the effects of non-nutritive additives (Gatlin et al. 1992; Zhang et al. 1999; Twibell et al. 2003). In mammalian studies it has been found that glycyrrhetinic acid (GA) promotes lipolysis and reduces subcutaneous fat deposition (Armanini et al. 2005; Luo et al. 2010). GA protects against diabetes complications by reducing fat peroxidation and increasing antioxidant activity in mammals (Kroes et al. 1997). Our previous study found that GA can reduce adipose tissue mass and plasma biochemical index of channel catfish (Jiang et al. 2012a; Jiang et al. 2012b). However, the underlying mechanism is still unknown, and warrants further in-depth studies. GA has traditionally been used as an anti-inflammatory in Chinese medicine through the mediating of tumor necrosis factor alpha (TNF-α) and NF-κB (Ishida et al. 2012). TNF-α is one of the most important cytokines secreted from adipose and liver tissue, which plays a significant role in the regulation of lipid metabolism, especially in fish (Liu et al. 2015). According to previous studies, TNF-α is a limiting factor of lipid depositional in gilthead sea bream (Saera-Vila et al. 2007). Furthermore, it also affects the lipocyte function of gilthead sea bream (Saera-Vila et al. 2007) and rainbow trout (Albalat et al. 2005) and inhibits the differentiation of adipocyte precursor in rainbow trout (Bouraoui et al. 2008). Therefore, TNF-α may be a potential regulator in improving lipid deposition. However, the relevance of TNF-α molecule to the pathophysiology of lipolysis in cultured fish, especially herbivorous fish, still remains unclear.

Lipoprotein lipase (LPL), a key enzyme which hydrolyzes triglycerides as very low-density lipoproteins (VLDL) and chylomicrons (CM), was inhibited by TNF-α both adipose tissue in vivo and preadipocytes in vitro. The negative effect of LPL expression by TNF-α through a functional OCT-1/NF-Y site in fish has been reported (Saera-Vila et al 2007). LPL, suppressed by TNF-α, which determined dietary lipids are partitioned towards storage or utilization (Saera-Vila et al. 2005), provided few free fatty acids (FFA) for storage by adipose tissue and liver (Mead et al. 2009). The cDNA of LPL and its regulation of activity and expression by dietary lipid levels from blunt snout bream *Megalobrama amblycephala* has been reported (Li et al. 2013), but little is known about its molecular mechanism by TNF-α in herbivorous freshwater fish.

Megalobrama amblycephala is an herbivorous freshwater fish popular to China. Due to its fast growth, tender flesh and high disease resistance, this species has been widely favored for aquaculture in China and other countries (Li et al. 2013). However, the artificial rearing of this species is often accompanied by the occurrence of fatty liver diseases, which results in a high mortality rate and poor growth (Park. 2010). Therefore, it is important to find an effective solution to reduce the lipid deposition of this fish.

This study was conducted to evaluate the effects of dietary GA levels on the lipid metabolism of *Megalobrama amblycephala* with special reference to TNF-α and LPL expression. The results obtained here may not only clarify the nature underlying the lipid metabolism disorder in fish, but also provide an approach to avoid and/or solve this problem.

Materials and Methods

Fish, feed, and diets

Juvenile *Megalobrama amblycephala* were obtained from FHYZ (the Fish Hatchery of Yangzhou, Jiangsu province, China) and acclimated to cage conditions for 15 days from a commercial diet to the basal diet. Then three hundred fish (initial average weight of 68.78 ± 1.13 g) were equally distributed into 12 cages (length: width: height, 1.5×1.2×1 m^3) at the rate of 25 fish/cage. Each diet was randomly assigned to four cages. Fish were hand-fed to apparent satiation three times daily (8:00, 12:00 and 16:30) for 8 weeks. During the feeding trial, dissolved oxygen was maintained above 4.8 mg/L by slow flowing water, and water temperature fluctuated between 24-30°C.

Three isonitrogenous (32.7% crude protein) experimental diets were formulated to contain increasing GA (purchased from Nanjing ZeLang Medical Technology Company Nanjing, China) at levels of 0 (control), 30, and 300 mg/kg, respectively. Formulation and proximate composition of the experimental diets are presented in Table 1. Diets were formulated to meet the nutritional requirements of this species according to Li et al. (2010).

All feed ingredients were ground into fine powder and blended with mixed oil (fish oil: soybean oil = 1:1). Initially, GA was added into a small part of the powered soybean meal, then to the rest of the powered soybean meal, and then added to the remaining ingredients. Finally, some water was added to produce soft dough. The dough was then pelleted using a pellet machine, and dried in a cool ventilated place at room temperature. All pellets were stored in a refrigerator at -20°C until use.

Table 1. Formulation and proximate composition of the basal diet (on fed basis).

Ingredients	*(%)*	*Proximate composition*	
Fish meal	8.00	Moisture	10.20
Soybean meal	30.00	Crude protein	32.70
Rapeseed meal	15.00	Crude lipid	5.13
Cottonseed meal	15.00	Ash	7.88
Fish oil	2.20	Gross energy (MJ/kg)	16.70
Soybean oil	2.20		
Wheat bran	5.00		
Wheat flour	19.60		
Ca(H2PO4)2	1.80		
Premix*	1.00		
Salt	0.20		
Total	100.00		

* Premix supplied the following vitamins (IU or mg/kg) and minerals (g/kg): Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1090 mg; Vitamin B5, 2000 mg; Vitamin B6, 500 mg; Vitamin B12, 1.6 mg; Vitamin C, 5000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; $CuSO_4 \cdot 5H_2O$, 2.0 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g.

Sampling and analytical procedures

At the end of the 8-week feeding trial, fish were fasted for 36 h (one day and two nights), then anesthetized in diluted MS-222 (tricainemethane sulfonate, Sigma, St. Louis, MO, USA) at a concentration of 100 mg/L (Jiang et al. 2012b). Final body weight of fish in each cage was determined, and weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated using the following formula:

Weight gain (WG) = (Final body weight – Initial body weight) *100 / Initial body weight

Specific growth rate (SGR) = (Ln (Final body weight) - Ln (Initial body weight))*100/56 Days

Feed conversion ratio (FCR) = Total diet fed / Total wet weight gain

Six fish from each cage (i.e. 24 fish per diet) were randomly collected for analysis of hepatosomatic index (HSI), viscera/body ratio (VBR), intraperitoneal fat ratio (IFR), and condition factor (CF).

Hepatosomatic index (HSI) = liver weight * 100 / body weight

Viscera / body ratio (VBR) = viscera weight * 100 / wet body weight

Intraperitoneal fat ratio (IFR) = intraperitoneal fat weight *100 / wet body weight

Condition factor (CF) = body weight * 100 / total body length ^{3}

In addition, blood from 12 fish, which were randomly sampled from each cage (3 fish per cage), was taken from the caudal vessels using heparinized syringes. Plasma from blood samples was centrifuged (2000×g at 4°C for 10 min) and was then stored at -20°C until further analysis (CHO, TG and NFFA were tested by kit from Nanjing Jiancheng Bioengineering Institute). Samples of liver and adipose tissue from three fish per cage were rapidly excised and frozen in liquid nitrogen and stored at -80°C until PCR analysis.

Total RNA extraction and Real-time PCR was conducted as in our earlier research (Li et al, 2013). Nucleotide sequences of primers, shown in Table 2, for the real-time PCR analysis, were designed using the Primer 5.0 Software, based on the cDNA sequences of *Megalobrama amblycephala* available in genebank.

Table 2. Primers used to assay gene expression by real-time PCR.

Target gene	*Accession number*	*Forward (5'-3')*	*Reverse (5'3')*
TNF-α	KU976426	TTACAGGCTGAGATTGACTA	GAAGAACATCCACGAAAA
LPL	AGQ42625	TCAAAGTCAGGCGTATGG	CTGGCTGTAGACGAAGTAAAT
β-Actin	AY170122.2	CGGACAGGTCACACCTTG	CGCAAGACTCCATACCCAAGA

TNF-α: Tumor necrosis factor alpha; LPL: Lipoprotein lipase.

Statistical analysis

All data for statistical analysis were subjected to one-way ANOVA followed by Dancan test using SPSS 19.0 (SPSS 19.0, Michigan Avenue, Chicago, IL, USA). Significance was determined at $P < 0.05$. All data are presented as means ± S.E.M. of 4 cage replicates.

Results

Growth performance and Biometric parameters

No mortalities were observed during the experiment. Final body weight, specific growth rate (SGR), and feed conversion ratio (FCR), all showed no significant difference ($P>0.05$) among treatments (Table 3). Weight gain (WG) in fish fed 300 mg/kg GA was lower than the other groups ($P>0.05$).

Hepatosomatic index (HSI), Viscera/body ratio (VBR), Intraperitoneal fat ratio (IFR), and Condition factor (CF) in fish fed diets containing various GA levels is shown in Table 4. There were no significant effects on VBR ($P>0.05$). However, HSI and CF decreased significantly ($P<0.05$) with increasing dietary GA levels, being lowest in 300 mg/kg GA group ($P<0.05$). IFR decreased significantly as dietary GA levels increased from 0 to 30 mg/kg ($P<0.05$) but showed little difference with further increasing GA levels ($P>0.05$).

Table 3. Growth performance of *Megalobrama amblycephala* fed diets containing various GA concentrations.

Group	*Initial body weight (g)*	*Final body weight (g)*	*Weight gain (%)*	*Specific growth rate*	*Feed conversion ratio*
Control	70.33±0.88	223.65±0.86	218.09±4.40	1.93±0.02	1.96±0.10
GA 30mg/kg	67.33±2.03	214.14±4.90	218.67±12.74	1.93±0.07	2.04±0.13
GA 300mg/kg	68.67±2.85	212.42±3.83	210.78±17.80	1.88±0.09	2.16±0.09

Values are presented as means of 4 replications.

Table 4. Biometric parameters of *Megalobrama amblycephala* fed diets containing various GA concentrations.

Group	*Hepatosomatic index (%)*	*Viscera/body ratio (%)*	*Intraperitoneal fat ratio (%)*	*Condition Factor (%)*
Control	1.27±0.07^{a}	12.22±1.15	1.90±0.18^{a}	2.41±0.05^{a}
GA 30mg/kg	1.15±0.05^{a}	11.26±1.11	1.54±0.15^{b}	2.33±0.06^{ab}
GA 300mg/kg	1.09±0.06^{b}	11.04±0.92	1.52±0.13^{b}	2.28±0.04^{b}

Values are presented as means of 4 replications.
Means in the same column with different superscripts are significantly different ($P<0.05$).

Plasma lipid metabolites

Data for plasma cholesterol (CHO), triglycerides (TG), and non- esterified free fatty acids (NFFA) levels are shown in Table 5. Plasma CHO, TG, and NFFA in different groups decreased significantly ($P<0.05$) with increasing dietary GA levels, the lowest occurring in fish in the 300mg/kg GA group ($P<0.05$).

Table 5. Blood biochemistry of *Megalobrama amblycephala* fed diets containing various GA concentrations.

Group	*Cholesterol (mg/dl)*	*Triglycerides (mg/dl)*	*Nonesterified Free Fatty Acids (umol/L)*
Control	129.33±8.55^{a}	148.95±6.05^{a}	504.92±132.56^{a}
GA 30mg/kg	104.69±9.44^{ab}	140.84±6.96^{a}	252.46±89.65^{ab}
GA 300mg/kg	98.44±5.35^{b}	114.95±3.03^{b}	126.23±21.65^{b}

Values are presented as means of 4 replications. Means in the same column with different superscripts are significantly different ($P<0.05$).

Tissue TNF-α and LPL expression

As can be seen from Figures 1 and 2, TNF-α expression increased significantly ($P<0.05$) with increasing dietary GA levels in both adipose tissue and liver. LPL expression decreased significantly ($P<0.05$) as dietary GA levels increased in adipose tissue. However, LPL expression in liver showed no significant difference ($P>0.05$) as dietary GA levels increased from 0 to 30 mg/kg but decreased significantly ($P<0.05$) with further increasing GA levels.

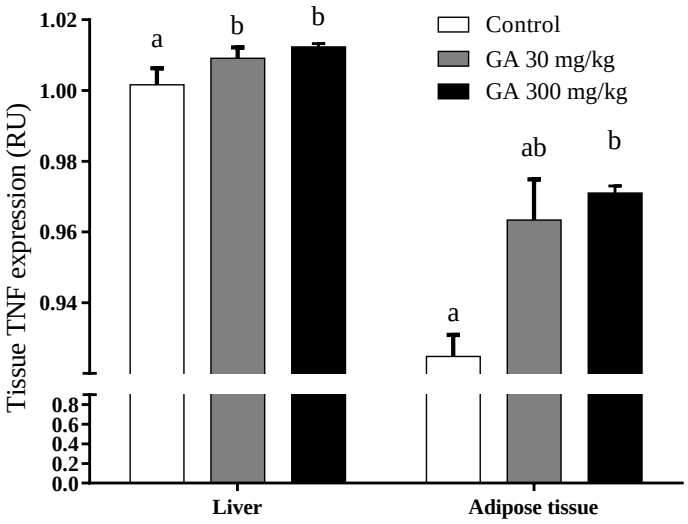

Figure 1. Tumour Necrosis Factor alpha tissue (TNF-α) expression of *Megalobrama amblycephala* fed diets containing various GA concentrations.
Data refers to values (Relative units, RU) found in the liver of the control group. Data represents the mean of 4 replicates. Bars assigned with different superscripts are significantly different (P< 0.05).

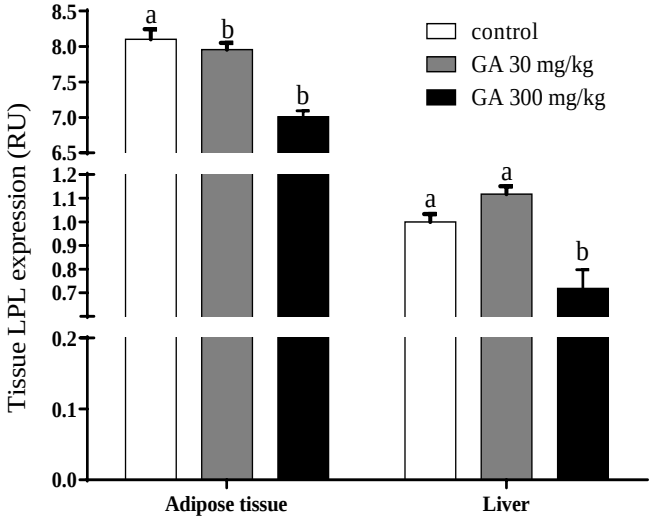

Figure2. Lipoprotein lipase tissue (LPL) expression of *Megalobrama amblycephala* fed diets containing various GA concentrations.
Data refers to the values (Relative units, RU) found in the liver of the control group. Data represents the mean of 4 replicates. Bars assigned with different superscripts are significantly different (P< 0.05).

Discussion

GA, the main active constituent of licorice root, has a hypolipidemic effect in rats (Panneerselvam et al. 2009) and decreases the body fat mass in human beings (Armanini et al. 2005; Armanini et al. 2002; Armanini et al. 2003). Dietary supplemented with, and cutaneous application of GA, showed lipolysis in mammals. Consequently, investigation of its function and molecular mechanism when supplemented in fish diets might give an indication of how to improve fish tissue lipid profiles. Our previous study found that GA could improve the lipid deposition and plasma biochemical index in channel catfish (Jiang et al. 2012a; Jiang et al. 2012b). There are few reports concerning the lipolytic effects of GA in herbivorous fish with the potential mechanisms unclarified.

It is generally recognized that animals fed GA diets usually show poor growth performance (Jiang et al. 2012a). However, in other studies, glycyrrhizin diets improved body growth of *Litopenaeus vannamei*. (Chen et al. 2010; Bai et al. 2010). In this trial, GA had no significant effect on fish growth. Similar results also were observed in channel catfish (Jiang et al. 2012b), and large yellow croaker (Xu et al. 2015). It seems plausible that GA affects growth by a comprehensive regulatory action. GA has negative effects on fish growth performance through enhanced immunization (Xu et al. 2015), increased antioxidation (Armanini et al. 2002), and promotes lipolysis (Luo & Ouyang, 2010). GA has positive effects on growth and increases the length of the fish (Jiang et al. 2012b) by stimulating food intake (Kroes et al. 1997). These conflicting reports could explain why growth performance in fish displays different trends at different time intervals.

In previous studies we found that feeding catfish (150-380 g) with 300 mg GA/kg body weight of GA significantly reduced IFR (Jiang et al. 2012b), and feeding catfish (1-8 g) with 300 mg GA/kg body weight of GA significantly reduced IFR and CF compared with the GA-free group (Jiang et al. 2012a). However, in this study, HSI and CF were affected in the 300 mg/kg GA group, and IFR was affected by diets containing 30 mg/kg GA of *Megalobrama amblycephala*. Based on these reports, we concluded that GA is beneficial in decreasing lipid deposition in both the liver and intraperitoneal fat tissue in fish, but the effective dose may be influenced by the size and species of fish.

Plasma cholesterol, triglycerides, and nonesterified free fatty acid concentrations in *Megalobrama amblycephala* in the present study, tended to decrease with increasing dietary GA levels, probably suggesting a lipolytic response to dietary GA levels. Similar results were observed in both rats (Panneerselvam et al. 2009) and humans (Armanini et al. 2002; Armanini et al. 2003). This result may also suggest that GA decreases body lipid deposition in fish by the mediation of plasma biochemical index. However, further in-depth studies are needed to elucidate this.

TNF-α is known as a restrictive factor in lipid deposition of fish and promotes lipolysis (Liu et al. 2015). It is one of the most important cytokines secreted from adipose tissue and plays a significant role in the regulation of lipid metabolism. Many studies have indicated TNF-α accelerates steatolysis in vitro through the activation of ERK1/2, p38 kinase, and JNK (Liu et al. 2015; Ryden et al. 2002). In the present study, tissue TNF-α expression of *Megalobrama amblycephala* increased with increasing dietary GA levels. This may indicate that GA promotes TNF-α expression. GA affected TNF-α and induced the expression and activity of MMP-9 through NF-κB pathway in cancer cells (Jayasooriya et al 2014). Similar results were observed in rat cells (Park, 2010). This indicates that TNF-α might be a target spot of GA in the regulation of fish lipid metabolism. However, further in-depth studies are needed.

LPL, which is a key enzyme, hydrolyzing triglycerides to provide FFA for storage by the adipose tissue and liver (Mead & Ramji, 2002), is inhibited by TNF-α both in vivo and in vitro (Bulló-Bonet et al. 1999; Panneerselvam et al. 2009; Ruan et al. 2003). According to Saera-Vila et al (2007), LPL mRNA expression and activity has been suppressed by TNF-α through a functional OCT-1/NF-Y site in fish. Contrary to the expression of TNF-α, tissue LPL expression of *Megalobrama amblycephala*, in the present study, decreased with increasing dietary GA levels, and may indicate GA regulated lipolysis through TNF-α to LPL expression.

In conclusion, dietary supplementation of 300 mg/kg GA had a positive effect in reducing fat deposition in both the liver and intraperitoneal fat tissue in *Megalobrama amblycephala* without significant side effects on growth. This beneficial effect can be ascribed to the regulation of TNF-α and LPL expression.

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