Original Article

An Assessment of Androgenic/Anti-androgenic Effects of GH Transgenic Carp by Hershberger Assay*

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Abstract

Objective To evaluate the androgenic and anti-androgenic effects of GH (growth hormone) transgenic carp in male rats.

Methods Hershberger assay was carried out in castrated male SD rats aged 4-5 weeks. Testosterone propionate (TP) (0.4 mg/kg BW) was administrated for a positive control, GH transgenic carp (3.0 g/kg BW)+TP (0.4 mg/kg BW), parental carp (3.0 g/kg BW) + TP (0.4 mg/kg BW), and flutamide (Flu) (3.0 g/kg BW) were used for negative controls, and vehicle was administered orally for a blank control. All groups were administrated for 10 consecutive days. At the end of the test, animals were anesthetized, then weights of accessory sex organ were measured. Serum testosterone (T), luteinizing hormone (LH), and Follicle-Stimulating Hormone (FSH) levels were detected.

Results The weights ratios of the accessory sex organs and body weights showed no significant differences between the solvent control and the GH transgenic carp-treated groups. Serum concentrations of FSH, LH, and T of the rats treated with GH transgenic carp + TP showed no significant changes, compared with those treated with TP only.

Conclusion GH transgenic carp does not have any androgenic agonist or antagonist properties in vivo screening tests.

Key words: GH gene transgenic carp; Hershberger assay; Androgenic properties

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INTRODUCTION

GH gene transgenic carp is a carp whose genome is inserted with grass carp GH genes by biotechnological methods. Its growth rate is increased by 15% and the forage utilization rate increased by 11.1%, compared with that of the control carp[1]. GH transgenic carp may be suitable for industrialized conditions. So breeding GH transgenic carps may have potential commercial uses as food, but its safety has hardly been evaluated either *in vivo* or *in vitro*. Chen[2] and Zhang et al.[3] have carried out conventional toxicological tests by using transgenic fish to feed animals. However, it is important to assess whether the normal endocrine system of rats is disrupted by the transgenic carps.

Hershberger assay was designed to screen the androgenic and myotrophic activity of steroids by

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assessing the ability of these molecules to induce growth in the levator animuscle, seminal vesicles, and prostate of weaned castrated rats[4]. Since 1998, this assay has also been recommended for the assessment of anti-androgenic activity in immature male rats[5]. A wide range of chemicals have been reported to have possible endocrine-disrupting activities in humans and animals[6-7]; therefore, the Organization for Economic Cooperation and Development(OECD) has proposed Hershberger assay as an *in vivo* screening test to detect androgenic and antiandrogenic properties[8].

In vivo data on the potential endocrine-disrupting properties of GH gene transgenic carp have not been available. We have performed Hershberger assay to detect possible androgenic agonist or antagonist properties and thus evaluate whether GH gene transgenic carps have endocrine-disrupting properties. It will provide relevant scientific evidence for food safety evaluation of GH transgenic carps.

MATERIALS AND METHODS

Materials

Testosterone propionate (TP) (purity 98%) was purchased from Zhongsheng Chemical Co. Ltd. (Beijing, China). Flutamide(Flu) (Lot：F9397-5G) was purchased from Beijing Kehaoda Bio-Technology Co. Ltd. (Beijing, China). GH transgenic carp and its parental carp were provided by Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Corn oil was bought from COFCO. (Beijing, China). The *T, FSH, and LH* ELISA kits were procured from Cayman and BIO-LAB INC (USA).

TP and Flu were dissolved in corn oil and used as a positive control for androgenic and anti-androgenic effects, respectively. GH transgenic carp and parental carp were vacuum freeze-dried and grinded into powder, then mixed and made into water suspension.

Animals and Housing Environment

Immature male SD rats, 4-5weeks old, were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) [license number: SCXK (Jing) 2006-0009]. The rats were housed in an animal room and maintained at 24±1 °C with a relative humidity of 50%±10% and an altering 12:12-h light-dark cycle. The rats were housed in stainless steel, wire-mesh cages with three per cage and fed with isoflavones diet and alfafa free diet (SAFD) (Beijing HFK Bio-Technology. Co. Ltd.). Food and tap water were provided *ad libitum*. This study was conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology and approved by the Medical Ethics Committee.

Hershberger Assay

Hershberger assay was performed in accordance with the current draft guidelines for the rodent Hershberger assay[5]. All animals were checked by a physical examination for clinical signs of ill health and observed for 7 days following their arrival. Then the rats were castrated and given 8 days for recovery. The rats were weighed, weight-ranked and randomly divided into seven groups, including the GH transgenic carp (3.0 g/kg BW) group, the GH transgenic carp (3.0 g/kg BW) plus TP(0.4 mg/kg BW) group, the parental carp (3.0 g/kg BW) group, the parental carp (3.0 g/kg BW) plus TP (0.4 mg/kg BW) group, the flutamide (3.0 mg/kg BW) positive control group, the TP (0.4 mg/kg BW) group as the negative control group, and the vehicle as the blank control group. The TP group was administered by subcutaneous injection in the back. The others were orally administered for 10 days consecutively.

The rats were weighed and killed in 24 h after the last administration. Blood samples were collected in plastic tubes and centrifuged for 10 min at 3 000 rpm at 4 °C to obtain sera. Sera were separated and stored at -20 °C until assay for FSH, LH, and T. Five androgen dependent accessory reproductive glands [the ventral prostate, seminal vesicles, levator plus bulbocavernosus muscles (LABC), Cowper's gland, and the glans penis] were excised to carefully remove excessive adhering connective tissues and fat, and weighed immediately.

T, FSH, and LH Measurement

The serum concentrations of T, FSH, and LH were measured with specific Enzyme-Linked Immuno Sorbent Assay (ELISA) kits. Tecan Sunrise read OD 450nm was used for T, FSH, and LH.

Statistical Analysis

SPSS17.0 Statistical System was used to analyze data. All data are presented as mean±standard error the mean (SEM). They were analyzed by one-way analysis of variance (ANOVA), which was followed by Dunnett's multiple comparison test. Differences were considered statistically significant when the *P* level was less than 0.05.

RESULTS

In comparison with the solvent control group, the castrated-immature, testosterone-treated male rats in all the treatment groups grew normally, and during the 10 d treatment with GH transgenic carp or carp, there were no significant changes in body weight (Table 1).

Table 1. Body Weight Changes among Different Groups at the Beginning and End of the Test ($\bar{x} \pm s$)

Groups	*n*	Body weight (g)	Final Body weight (g)	Gain weight (g)
GH gene transgenic carp (3 g/kg BW)	9	145.22 ± 1.64	256.2 ± 26.6	110.99 ± 25.83
GH gene transgenic carp (3 g/kg BW)+ TP (0.4 mg/kg BW)	9	145.11 ± 2.66	257.4 ± 17.0	112.37 ± 18.06
Carp (3 g/kg BW)	9	147.11 ± 4.91	253.0 ± 16.7	105.87 ± 20.04
Carp (3 g/kg BW) + TP (0.4 mg/kg BW)	9	145.56 ± 3.97	261.6 ± 21.3	116.06 ± 23.16
TP (0.4 mg/kg)	9	145.00 ± 4.18	264.8 ± 16.9	119.79 ± 18.11
Flu (3 mg/kg BW) + TP (0.4 mg/kg BW)	9	145.33 ± 1.11	265.1 ± 13.9	120.73 ± 13.87
Solvent Control	9	144.33 ± 6.50	256.0 ± 17.2	110.71 ± 16.92

Note. Values are expressed as mean±SEM for nine animals.

The organ weight ratios of the androgen-dependent accessory reproductive glands organ are shown in Table 2. The weights ratios of ventral prostate, seminal vesicle, LABC, glans penis, and Cowper's glands were significantly increased in the TP group, the GH transgenic carp plus TP group and the carp plus TP group, as compared with those of the solvent control group and the carp control group (P<0.05). The organ weights ratios in the GH transgenic carp group or the carp group had no significant increase, compared with those of the solvent control group (P>0.05). The weight ratios of ventral prostate, seminal vesicle, LABC, Glans penis, and Cowper's glands were significantly decreased in the Flutamide plus TP group, compared with those of the TP control group. The organ weights ratios of the GH transgenic carp plus TP group or the carp plus TP group had no significant differences, as compared with those of the TP control group(P>0.05).

Table 2. Weights of Accessory Sex Organs of Castrated Rats among Different Groups after Treatment (%)($\bar{x} \pm s$)

Groups	*n*	Ventral Prostate /BW(mg/g BW)	Seminal Vesicle /BW(mg/g BW)	LABC/BW(mg/g BW)	Glans Penis /BW(mg/g BW)	Cowper's Glands /BW(mg/g BW)
GH gene transgenic carp (3 g/kg BW)	9	7.72 ± 4.58^{a}	16.68 ± 3.50^{a}	51.11 ± 7.70^{a}	17.21 ± 2.92^{a}	2.62 ± 1.10^{a}
GH gene transgenic carp (3 g/kg BW)+TP (0.4 mg/kg BW)	9	27.75 ± 7.39^{bc}	66.70 ± 14.91^{bc}	80.84 ± 16.04^{bc}	29.08 ± 3.25^{bc}	8.58 ± 1.84^{bc}
Carp (3 g/kg Bw)	9	8.16 ± 4.27^{a}	16.92 ± 4.14^{a}	45.38 ± 9.11^{a}	16.48 ± 1.42^{a}	2.62 ± 1.48^{a}
Carp (3 g/kg BW)+TP (0.4 mg/kg BW)	9	29.56 ± 9.28^{bc}	50.94 ± 11.72^{bc}	80.34 ± 14.27^{bc}	26.03 ± 2.96^{bc}	8.48 ± 3.00^{bc}
TP(0.4 mg/kg BW)	9	26.34 ± 8.51^{bc}	59.33 ± 13.49^{bc}	87.07 ± 16.92	25.66 ± 3.30^{bc}	10.33 ± 2.43^{bc}
Flu (3 mg/kg BW)+TP (0.4 mg/kg BW)	9	11.30 ± 2.28^{ab}	33.13 ± 7.39^{abc}	60.80 ± 8.96^{abc}	18.07 ± 4.16^{a}	4.03 ± 1.48^{a}
Solvent control	9	7.42 ± 2.06^{a}	11.23 ± 3.23^{a}	48.99 ± 13.43^{a}	15.65 ± 2.47^{a}	1.98 ± 1.35^{a}

Note. Values are expressed as mean ± SEM for nine animals. ^{a}P<0.05 as compared with the value for the TP control group; ^{b}P<0.05 as compared with the value for the solvent control group (Dunnett's multiple comparison test); ^{c}P<0.05 as compared with the value for the carp control group (Dunnett's multiple comparison test).

No statistically significant differences were observed among the GH gene transgenic carp plus TP group, the parental carp plus TP group and the TP control group for the serum concentrations of T, FSH, and LH. The serum concentrations of LH, FSH were significantly increased in the Flutamide plus

TP group, compared with the TP control group (P<0.05) (Table 3).

Table 3. Plasma Concentrations of FSH, LH, and Testosterone in Different Groups ($\overline{x} \pm s$)

Groups	*n*	LH (mIU/mL)	T (ng/mL)	FSH (ng/mL)
GH Gene Transgenic Carp 3 g/kg BW	9	0.374±0.063 [a]	0.68±0.32 [a]	0.591±0.069 [a]
GH Gene Transgenic Carp 3 g/kg BW + TP (0.4 mg/kg BW)	9	0.257±0.048[b]	4.58±0.62[b]	0.471±0.045[b]
Carp 3 g/kg BW	9	0.376±0.054[a]	0.77±0.61[a]	0.586±0.039[a]
Carp 3 g/kg BW + TP (0.4 mg/kg BW)	9	0.233±0.050[b]	4.52±0.62[b]	0.471±0.059[b]
TP (0.4 mg/kg)	9	0.279±0.032[b]	4.97±0.77[b]	0.492±0.036
Flu3mg/kg BW+TP (0.4 mg/kg)	9	0.607±0.081[ab]	4.08±0.58[b]	0.721±0.046[ab]
Solvent Control	9	0.366±0.059[a]	0.67±0.14[a]	0.583±0.067[a]

Note. Values are expressed as mean±SEM for nine animals. T=testosterone. [a]P<0.05 as compared with the value for the TP control group; [b]P<0.05 as compared with the value for the solvent control group (Dunnett's multiple comparison test).

DISCUSSION

Transgenic carps containing grass carp growth hormone (GH) gene have improved growth characteristics. Although genetically modified (GM) animal foods are beneficial for ensuring a constant supply of foodstuffs, their safety remains doubtful. Therefore, it is necessary to test their safety before they are released on the market. The researches on the safety of transgenic fish have been conducted with conventional toxicological methods. For examples, Chen Kaijian[2] used grass carp with Hu-α-IFN gene to feed rats, and found no significant difference in haematological index, morphology and histopathological examination of the main organs between the treatment group and the control group. Zhang Fuying[3] fed mice with "all fish" gene transgenic carps and found that mouse weight gain, blood routine examination, and histopathology had no abnormal change. However, studies on the endocrine disruption effects of GH transgenic crap have not been reported so far. This endocrine disturbance assay has its superiority over other in vivo procedures, with its simplicity, time-saving and relative specificity in directly detecting androgenic/antiandrogenic effects or estrogen/antiestrogen. We have already explored the potential estrogen-like activity of GH transgenic carp, and no estrogenic effect has been shown by uterotropic assay[9].

Hershberger assay was used to examine the endocrine disruptive effects of GH transgenic carp based on the responses of androgen-dependent tissues and hormones in castrated immature rats. In this assay, androgens by subcutaneous injection were usually absorbed completely and rapidly, but tested compounds were often orally administered to male rats to make the exposed pathway similar to the way of human's exposure to GH transgenic carp. Flutamide has been widely used in Hershberger assay as a positive anti-androgen control. In fact, flutamide as an androgen receptor antagonist can bind to the androgen receptor and effectively block the recognition of androgens, leading to the change of androgen-dependent tissues[10] and serum hormone levels[11]. This study showed that development of androgen dependent accessory reproductive glands of the Flu group seriously lagged and serum concentrations of FSH and LH were elevated. The obvious androgenic antagonist effect suggested that the castration surgery and the test model were successful. Testosterone has been found to have an androgenic effect by increasing the accessory reproductive organ weight, which has been studied by using both castrated immature and adult male rats[12]. Our results also showed that testosterone could increase the weights of seminal vesicles and Cowper's glands. Since the sensitivity of the Hershberger assay in the case of using non-castrated rats was lower than that in the case of using castrated rats, the castrated rats were selected in the experiment. No significant changes in accessory sex organ weights ratios were observed between the GH transgenic carp-treated groups and the solvent controls. The results suggest that the accessory sex organs of normal growing rats may be not influenced by GH transgenic crap.

In addition, serum FSH and LH levels may be useful endpoints in Hershberger assay. Flutamide significantly increased serum FSH and LH levels, but serum testosterone levels were relatively constant. In this study, the serum concentrations of testosterone

showed no significant changes in treatment with GH transgenic carp Plus TP, as compared to the TP-treated group. These results indicate that GH transgenic carp has no anti-androgenic effects on the hypothalamus-pituitary axis and the accessory sex glands, and that GH transgenic carp has neither androgenic agonist nor antagonist properties.

In summary, GH transgenic carps have no androgenic agonist or antagonist activities for rats. In further studies, it is necessary to detect antithyroid activity of animals which they eat GH transgenic carps, for the antithyroid activity may affect pubertal development of animals via changes in gonadotropins, prolactin, or hypothalamic function.

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