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Measurement of biological activity of somatotropin in hypophysectomized rats

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KEY WORDS somatotropin; biological assay; hypophysectomy; growth plate; body weight; recombinant DNA; Wistar rats

AIM: To develop a method for measurement of biological activity of recombinant DNA-derived somatotropin (rhGH). **METHODS**: The effects of varying the route, frequency and period of administration of GH, the sex of test animals on the biological responses, body weight gain (BWG), and tibial epiphyseal width (TEW), of hypophysectomized (Hypox) rats were compared, respectively. 4-d BWG, 6-d BWG, and 6-d TEW tests were carried out simultaneously in the same group of Hypox rats to determine the biopotency of GH preparations according to a parallel line bioassay (6-point assay). The final result was chosen from the test which had smaller values for the index of precision (λ) and the average rate of fiducial limits (ARFL) than other tests. **RESULTS**: No significant differences in the responses between male and female rats, between *sc* and *im*, once daily and twice daily injections of bGH were found. But the BWG and TEW of Hypox rats injected with 0.045 and 0.135 IU·d^{-1} of bGH for 6 d were significantly greater than that for 4 d. Both 4-d BWG test and 6-d BWG test in the range from 0.020 to 0.500 IU·d^{-1} had values for $\lambda = 0.0660$ and 0.1747, and for $r = 0.9000$ and 0.9237, respectively. Three estimates of rhGH preparation compared with the International Standard for somatotropin (IShGH), 4.6132, 3.9829, and 4.8023 IU/ampoule, were obtained separately from 4-d BWG test, 6-d BWG test and 6-d TEW test. And the result from 6-d BWG test was reported finally because it had smaller values for λ and ARFL (0.0608 and 37.907 %) than other two tests. **CONCLUSION**: Both BWG test and TEW test can be carried out simultaneously in the same group of Hypox rats. 6-d BWG test seemed to be more suitable for potency determination of GH preparations than 4-d BWG test and 6-d TEW test.

The whole procedures for body weight gain (BWG) test and tibial epiphyseal width (TEW) test were described in European Pharmacopeia[1] (EP) and were widely used to measure the biological activity of GH preparations[2,3]. The BWG test and TEW test in the EP are separate to use. The number of injection and the range of doses of GH preparations in these 2 methods are also different. In order to save the hypophysectomized (Hypox) rats that are not easy to prepare, and to make the successful rate of the experiment higher, an attempt to carry out both BWG test and TEW test for determining the biopotency of GH in the same group of Hypox rats was made, which has not been reported in previous literature. For this, some principal experimental factors that may have influences on the BWG and TEW of Hypox rats were investigated firstly.

MATERIALS AND METHODS

Drugs International Standard for bovine growth hormone (ISbGH) and somatotropin (IShGH) for bioassay, with an assigned biopotency of 1000 IU·g^{-1} and 4.4 IU/ampoule, respectively, were kindly supplied by the WHO. Recombinant DNA-derived somatotropin (rhGH), with a biopotency of 4 IU/ampoule, was kindly supplied by Laboratories Seroro SA, Switzerland. Human serum albumin (hSA) was purchased from Institute of Biological Products, Shanghai, China.

Preparation of GH solution Serial solutions of bGH, hGH, and rhGH were prepared with normal saline containing 0.1% hSA, divided into daily doses stored at −20 ℃.

Hypox rats Wistar rats, 70 - 80 g, 26 - 28 d old, were hypophysectomized by the parapharyngeal approach[4]. After control interval of 12 - 14 d, Hypox rats whose body weight (BW) did not increase or decrease beyond 10 % of original BW, amounting to 60 - 65 % in our experiments, were injected sc or im with GH for 4 or 6 d. Twenty-four h (once daily) or 16 h (twice daily) after the last injections, the rats were killed with an overdose of urethan. At autopsy the sella turcica was

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examined grossly. Only those data from rats with complete hypophysectomy were accepted.

Body weight gain (BWG) BW of rats were registered at 08:30. The net BWG was determined daily by subtracting the BW on the 1 day of GH injection from the BW on subsequent days

Tibial epiphyseal width (TEW) The outside of the proximal tibial epiphysis was split in the sagittal plane to expose the growth plate. The bone parts were labelled, and immersed in acetone for 6 min, and washed in water for 3 min, then placed in a fresh 2 % $AgNO_3$ for 2 min, rinsed again in water. Under water they are exposed to a strong light until the calcified portions appeared dark-brown, and immersed in 10 % sodium thiosulfate for 30 s, then stored in 80 % ethyl alcohol. Finally, a slice of 0.5 mm thick was cut out of the exposed part of the epiphysis. The width of the uncalcified growth plate were measured under the low power (4 × 10) of the microscope provided with an eyepiece micrometer. Ten readings were made at 10 sites over its full length, and the average was regarded as the response in TEW test.

Sex and route of injection Four groups of Hypox ♂ rats were injected daily with bGH 0.0325 or 0.0650 IU for 6 d by sc or im route. So did 4 groups of Hypox ♀ rats.

Frequency of injection Thirty-two Hypox ♂ rats were divided equally into 4 groups. Two total doses of 0.065 and 0.130 IU·d^{-1} of bGH were injected sc to 2 groups 0.5 mL once daily (08:30 with bGH, 16:00 with 0.5 mL saline), and to the others 0.5 mL twice daily (08:30, and 16:00) for 4 d.

Period of injection Three groups of ♂ Hypox rats received saparately a twice daily sc injection of bGH 0.015, or 0.045, or 0.135 IU·d^{-1} for 4 d. Another 3 groups received the same treatment but for 6 d.

Dose-response relationship bGH (4, 20, 100, 250, and 500 IU·L^{-1}) was injected sc to ♂ Hypox rats 0.5 mL twice daily for 8 d. The λ and γ were computed. The control group received equal amounts of saline for 8 d.

Potency determination A parallel line bioassay (6-point assay) was employed in determination of the potency of bGH, hGH, and rhGH preparations. GH 15, 45, and 135 IU·L^{-1} were injected *sc* to ♂ Hypox rats 0.5 mL twice daily for 6 d. The correlation coefficient (r) and the index of precision (λ_1) of each lg dose-response curve, and the index of precision (λ_2) of 2 lg dose-response curves in the 6-point bioassay were computed. Meanwhile, The parallelism and linearity of the curves were examined.

Statistical analysis The λ (s/b, s is pertaining to a departure from linearity[5], b is the slope of curve, or the combined slope of 2 curves in the 6-point bioassay), r, parallelism and linearity of the curve were determined by ANOVA. An estimate of potency (Pt) for each GH preparation, and its average rate of fiducial limits [ARFL, equal to (upper limit-lower limit)/2·Pt] were calculated[5]. The t test was used for other statistical calculations.

RESULTS

There were no significant differences between the mean BWG or TEW in the 2 comparable groups of bGH in relation to the sex, the route and frequency of daily injections (Tab 1, 2).

In response to 0.045 and 0.135 IU·d^{-1}, mean BWGs and TEWs were greater in the 6-d injection

Tab 2. Effect of frequency of injection of bGH for 4 d on BWG and TEW of Hypox ♂ rats. $\bar{x} \pm s$. $^aP > 0.05$ *vs* once daily, number of rats in parentheses

	Total dose/IU	
	0.26	0.52
Body weight gain		
Once dailly	6.9±2.3 (8)	8.8±1.5 (8)
Twice daily	7.3±2.4 (8)[a]	9.1±3.8 (8)[a]
Tibial epiphyseal width		
Once dailly	10.2±3.2 (8)	12.7±2.3 (8)
Twice daily	9.8±2.8 (8)[a]	11.4±3.6 (7)[a]

Tab 1. Effect of sex and route of injection of bGH for 6 d on BWG and TEW of Hypox rats. $\bar{x} \pm s$. $^aP > 0.05$ *vs* the same sex and the different routes, $^dP > 0.05$ *vs* the opposite sex and the same routes. Number of rats in parentheses.

Total dose/IU	Male rats		Female rats	
	sc	im	sc	im
BWG				
0.195	8.5±2.0 (7)	9.2±2.2 (7)[a]	9.1±1.5 (5)[d]	6.4±1.6 (5)[ad]
0.390	11.4±2.8 (6)	9.6±3.2 (8)[a]	12.0±3.1 (4)[d]	13.1±3.9 (6)[ad]
TEW				
0 195	9.7±1.8 (6)	9.8±2.0 (7)[a]	9.4±2.5 (5)[d]	8.9±1.7 (5)[ad]
0.390	10.2±0.9 (6)	10.2±2.1 (8)[a]	10.1±3.8 (4)[d]	10.0±2.3 (6)[ad]

period groups than in the 4-d period groups ($P < 0.05$). In the control groups, the TEW of Hypox rats were markedly greater than BWGs with the same injection periods (Tab 3).

In all 3 BWG tests, the λ in the range of 0.020 – 0.500 IU·d^{-1} were smaller than that in the range of 0.004 – 0.250 IU·d^{-1}. With injection period going up from 4 d to 8 d, the λ increased progressively, reaching a value of 0.3758 on d 9, indicating 8-d BWG test was less precise than 4-d and 6-d BWG tests. In addition, the r, 0.9000 and 0.9237, of the curves in 4-d BWG test and 6-d BWG test also indicated that these 2 curves were linear in the range of 0.020 – 0.500 IU·d^{-1} (Tab 4).

Tab 4. Index of precision (λ) and correlation coefficient (r) for the lg dose-response curves. $n = 5$, $\bar{x} \pm s$.

	Period/d 4	6	8
Control (saline)	−0.2±0.2	−0.3±1.0	−0.5±1.1
Dose/IU·d^{-1}			
0.004	6.9±1.7	6.7±1.0	9.2±2.1
0.020	9.0±3.5	10.3±1.9	12.9±1.8
0.100	15.0±2.9	16.2±3.0	19.2±3.3
0.250	18.9±1.0	21.7±2.3	28.9±2.1
0.500	21.0±1.5	24.6±2.4	34.6±3.1
Dose ranges 0.004 – 0.250			
r	0.8820	0.9289	0.9129
λ	0.5018	0.2171	0.7171
Dose ranges 0.020 – 0.500			
r	0.9000	0.9237	0.9389
λ	0.0660	0.1747	0.3758

Of the lg dose-response curves, the mean r and λ were, respectively, 0.7857 and 0.1885 in the three 4-d BWG test, 0.8790 and 0.0426 in the three 6-d BWG tests, 0.8376 and 0.3731 in the TEW tests. 6-d BWG test for each GH preparation had better values for r and λ than other two tests. Examination of curves for the GH preparation within the 6-point bioassay did not show any statistically significant departures from parallelism. But in one assay of ISbGH against IShGH with 6-d TEW test, a departure from linearity ($P < 0.05$) was found. Three 6-d TEW tests obtained greater λ_2, 0.3740 – 0.5134; and three 4-d BWG tests obtained greater values for ARFL, ranging from 50.84 % to 89.36 %, while the λ_2 and ARFL in three 6-d BWG tests were smaller ranging from 0.0109 – 0.0677 and from 37.90 % – 48.22 %, respectively (Tab 5).

DISCUSSION

The effect of the sex of test animals on the responses had been studied earlier. Geschwined *et al*[6] reported the male rat was perfectly comparable to the female used as usual in the bioassay of GH with respect to sensitivity, maximal response and slope of the dose-response curves. The result in the present investigation confirmed their views.

The effect of im administration of GH on the BWG and TEW of Hypox rats has not been reported. Previous studies had proved that there were no significant differences in responses between ip, iv, and sc administration of GH[7]. The same conclusion was reached from a comparison of the effects of im and sc injection of bGH at two dose levels in the present investigation. But because of being easy to operate, sc injection route was chosen in this modified procedure.

The effect of the frequency of injection on BWG of Hypox rats was studied in detail by Jansson *et al*[8] and Groesbeck *et al*[9]. Jansson *et al* using BWG test found that when the daily doses of hGH of 8 and 32

Tab 3. Effect of period of sc injection of bGH on the BWG and TEW of ♀ Hypox rats. Number of animals is in parentheses. $\bar{x} \pm s$. [a]$P > 0.05$ *vs* 4 d, [b]$P < 0.05$ *vs* 4 d.

bGH dose IU/d	Body weight gain 4 d	6 d	Tibial epiphyseal width 4 d	6 d
Control	−0.4±2.2 (5)	−0.5±2.4 (5)	4.8±0.7 (5)	5.2±0.5 (5)
0.015	6.5±3.2 (7)	6.6±0.7 (4)[a]	7.3±0.6 (7)	7.6±1.4 (4)[a]
0.045	6.9±3.4 (6)	12.4±3.6 (4)[a]	8.9±1.5 (6)	11.6±0.9 (4)[b]
0.135	11.0±3.7 (6)	18.4±4.6 (4)[b]	9.5±2.3 (6)	12.4±1.1 (4)[b]

Tab 5. Correlation coefficient (r) and index of precision ($\lambda 1$) for a lg dose-response curve, index of precision ($\lambda 2$) for 2 lg dose-response curves in 6-point bioassay. $\bar{x} \pm s$. Results of rhGH in comparison with IShGH in parentheses.

Preparations	Tests	Rats	Dose levels/IU·d^{-1} 0.015	0.045	0.135	r	$\lambda 1$	$\lambda 2$	Pt	ARFL
bGH	4-d BWG	4	5.1±1.3	8.2±3.8	12.3±3.5	0.7412	0.1052	0.3325	1.1111	89.367
	6-d BWG	4	6.6±0.7	12.4±3.7	18.4±4.6	0.8519	0.0101	0.0109	1.1946	49.355
	6-d TEW	4	7.6±1.4	11.6±0.9	12.4±1.1	0.8365	0.5495	0.5134	0.9018	47.124
hGH	4-d BWG	6	4.7±2.2	7.4±3.3	12.5±4.1	0.7079	0.4471	0.3325	3.9600	85.936
	6-d BWG	6	4.6±3.2	11.5±4.3	18.3±2.9	0.8682	0.0117	0.0109	3.6832	48.222
	6-d TEW	6	8.6±1.1	9.9±1.1	13.8±1.8	0.8289	0.4865	0.5134	4.8792	47.737
rhGH	4-d BWG	6	3.9±1.7	8.7±2.4	13.7±1.9	0.9079	0.0131	0.2803	4.6132	50.844
								(0.0624	4.2183	51.561)
	6-d BWG	6	7.0±1.6	11.1±1.7	16.2±2.0	0.9168	0.1060	0.0608	3.9829	37.907
								(0.0677	3.1777	43.917)
	6-d TEW	6	9.0±1.5	11.0±1.2	13.4±0.8	0.8473	0.0834	0.3740	4.8023	44.361
								(0.4114	5.4238	49.168)

μg were given *sc* to male SD Hypox rats on 2 occasions, BWG were significantly higher compared with giving the daily dose on 1 or 8 occasions. Groesbeck *et al* reported that cumulative BWG of female SD Hypox rats at the 50 μg hGH per day was significantly greater on the four times daily injection regimen than on the two times daily regimen on d 2 – 5. However, similar results did not come out of our experiments. No significant differences in both BWG and TEW of the male Wistar Hypox rats between the twice daily and once daily injection of bGH were found. The reasons why there were discrepancies in the results described above might be due to difference in the age and strain of the animals, lack of standardization of time periods between injections, injection of GH from different species, and so on.

In TEW test, the period of injection in earlier studies was always 4 d. According to the report by Greenspan *et al*[7], 4 or 5 d injection periods produced a satisfactory response, shorter injection period (< 4 d) was unsatisfactory in the response; increasing the injection period did not add to the sensitivity of the response. However, the results from the present experiment (Tab 3, 5) clearly showed that the mean TEWs of Hypox rats injected GH for 6 d were significantly greater than that for 4 d, indicating that the range of GH increased in the 6-d TEW test. This can be good for arranging doses in 4-d BWG, 6-d BWG, and 6-d TEW tests in the same group of Hypox rats.

In BWG test, the range of injection period was quite large from 4 d[1] to 29 d[9]. Groesbeck *et al*[9] reported that 9-d period of injection of hGH 2 or 4 times daily at dose of 10 and 50 μg·d^{-1} would result in hGH antibody formation and growth retardation. They suggested that injection period for hGH rather than bGH should be limited to 7 d. For bGH, the results in our experiments (Tab 4) proved that the values for λ in the 4-d or 6-d BWG test were markedly smaller than that in 8-d BWG test. This was because 8-d BWG test produced a larger standard diviation (s).

The results in our experiment (Tab 5) also demonstrated that as long as the range of doses was arranged properly, both BWG test and TEW test can be carried out simultaneously in the same group of Hypox rats. Evans *et al*[10] once made a comparison between TEW and BWG in the same group of Hypox rats injected with GH for 4 d and found that the lg dose-response curve over the range from 4 to 200 mg·d^{-1}, was a straight line in TEW test, but was a quite irregular line in BWG test. Thorngren *et al*[11] reported that compared with the accumulated longitudinal bone growth of proximal tibia in Hypox rats given the total doses of 250 – 4000 μg of bGH for 10 d, the BWG at the end of the injection period had a poor index of precision ($\lambda = 0.589$) and the correlation coefficient was much lower ($r = 0.620$). However, in the present investigation (Tab 5), of lg dose-response curves for GH preparations in both 6-d BWG and 6-d TEW tests, the r ranged from 0.8289 to

0.9168, and λ ranged from 0.0101 to 0.5495. Here the selection of the range of doses was a vital factor. The range of doses of three GH preparations in our experiment was at the lower dose levels (0.015 – 0.135 IU · d^{-1}), which proved to be practicable. But, on the other hand, if the dosage of GH was too low, an effective response can not be produced, especially in TEW test (Tab 3). Therefore, the arrangement of doses of GH was required to be suitable not only for BWG test but also for TEW test, which may be a shortcoming of this modified method. Besides, final result was selected by the values for λ and ARFL because the smaller the λ and ARFL, the more precise the bioassay. 6-d BWG test had lower values for λ2 and ARFL (0.0608 and 37.907 %, according with the demand on the fiducial limits of error of the estimated potency of somatotropin in EP[1]) in measuring the biopotency of rhGH preparation in comparison with IShGH, so its result, 3.9829 IU/ampoule, should be reported definitively.

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生长激素在去垂体大鼠体内的生物活性测定

R977.1

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关键词 生长激素; 生物测定; 垂体切除术; 生长板; 体重; 重组 DNA; Wistar 大鼠

目的: 建立测定生长激素(GH)在体生物活性的方法. **方法**: 以去垂体大鼠体重增长(BWG)和胫骨骺软骨板宽度(TEW)为指标, 观察动物性别、给药途径、次数和周期不同对效应的影响; 同时进行 4-d BWG, 6-d BWG 和 6-d TEW 法, 测定 GH 的效价(平行线 3×3 设计). **结果**: ♀和♂ sc 和 im 给药以及每日给药 1 次和 2 次的 BWG 和 TEW 差异无显著意义. 给药 6 d 比给药 4 d 引起较大的 BWG 和 TEW ($P < 0.05$). 4-d BWG 法和 6-d BWG 法在 0.020 – 0.500 IU · d^{-1} 有较好的 λ 值(0.0660 和 0.1747)和 r 值(0.9000 和 0.9237); 4-d BWG、6-d BWG 和 6-d TEW 法测得 rhGH 的效价为 4.6132、3.9829 和 4.8023 IU/amp. 6-d BWG 法有较小的 λ 值和较低的 ARFL 值. **结论**: 可在同一组去垂体大鼠体内同时用 4-d BWG、6-d BWG 和 6-d TEW 法测 GH 活性, 以 6-d BWG 法较好.