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Assessment of immune function after short-term administration of recombinant human growth hormone in healthy young males.

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Abstract

It is reported that in both elite and recreational athletes Growth Hormone (GH) is the most commonly used drug aimed at improving sport performance. **Aim:** The purpose of the present study was to evaluate the immunomodulatory effects of short-term administration of recombinant human GH (rhGH) in healthy young males. NK cell number, activity and phenotype, T cell number, cytokine production of IL-2, IL-4, IL-6, IL-10, TNF- α and IFN- γ and CD4⁺/CD8⁺ ratio with particular attention to the possible correlation to IGF-I production were investigated. **Methodology:** 30 healthy young males (27 \pm 9 yrs) were randomly assigned to either placebo (n = 15) or drug (rhGH) 1mg/day groups (n = 15) with daily injection for seven days. IGF-I serum concentration and flow cytometry data were generated at baseline (day 0) and days 8, 15, 22 and 29 post injection. Data was analysed using a General Linear Model with repeated measures with a Bonferroni correction factor and significance set at $p \leq 0.05$. **Results:** The T cell cytokine secretion profile indicated a Th1 - Th2 shift. Interestingly, a significant difference ($p \leq 0.05$) of IL-10 mean fluorescence is noted from day 15 (P= 35.14; \pm 19.93, rhGH = 26.63; \pm 16.39) to day 22 (P= 61.32; \pm 20.41, rhGH= 74.99; \pm 46.91) and to day 29 (P= 101.98; \pm 67.25, rhGH= 107.74; \pm 122.58). Serum IGF-I concentration (ng/mL) increased significantly ($p \leq 0.01$) on day 8 (0.48 \pm 0.78) after injections compared to baseline (0.31 \pm 0.07) as well at day 15 (0.33 \pm 0.06), 22 (0.29 \pm 0.05) and 29 (0.29 \pm 0.06). In conclusion, the cytokine secretion spectrum is affected by short-term rhGH administration in healthy young males being suggested to have induced CD4⁺ T lymphocytes production of IL-10.

Key words: Exogenous human growth hormone; Cytokines; Killer cells, natural; Doping in sports, insulin-like growth factor I

1. Introduction

Several factors contribute to the reasons why both top level and amateur athletes use banned substances and include prospects of increased financial gain, the desire to become famous and a raising of social status (Aquino Neto, 2001). Despite the widespread belief that administration of growth hormone (GH) will enhance sport performance, there is little supportive data to match this belief. Although it is well known that GH induces hepatic IGF-1 secretion, little is known regarding its biological effects on immune function and effects on the sporting performance of healthy individuals. GH has been banned in sports competition and training and is classified as a prohibited substance within section S2 (Peptide hormones, growth factors and related substances) of the list generated by the world anti-doping agency (WADA).

Previous studies have shown that GH alters natural killer (NK) cell function. Interestingly, NK cells activity has been found to be significantly impaired in GH deficient (GHD) patients (Crist, Peake, Mackinnon, Sibbitt, & Kraner, 1987; Sneppen, Mersebach, Ullum, & Feldt-Rasmussen, 2002). An 18 month randomized, placebo-controlled, double-blinded trial study (n=110) showed that both NK cells total number, and subtype NK cells (CD16⁺) were reduced in GHD patients compared to controls. (Sneppen, et al., 2002).

Peripheral blood NK cells can be subdivided into CD56^{dim} CD16⁺ and CD56^{bright} CD16⁻ NK cells (Caligiuri, 2008), which denote the normal and low cytotoxic activity respectively. Such phenotypic properties are responsible for unique functional attributes that directly impact on the human immune response (Cooper et al., 2001; Caligiuri 2008).

Other cells do express GH and IGF-I receptors and include B lymphocytes, macrophage, T lymphocytes and granulocytes precursors (Auernhammer & Strasburger, 1995). In GHD

patients suffering from hypopituitarism, they demonstrate immunological impairment parameters such as increased basal plasma levels of tumour necrosis factor- α (TNF- α - 220%) and IL-6 by 340% (Serri et al., 1999). Other studies have shown that blood TNF- α levels are significantly higher in children with GHD than the controls, and long-term therapy with rhGH was effective at reducing such levels (Andiran & Yordam, 2007). *In vitro* administration of exogenous GH has been shown to improve a variety of immune cell functions including B lymphocyte antibody production and responses (Kimata & Yoshida, 1994), NK activity (Stephenson, Lee, Bailey, Shepherd, & Melling, 1991), macrophage activity (Gaytan, et al., 1994), T lymphocytes and neutrophil functions (Fu, et al., 1992). The CD4⁺ T helper lymphocytes have a regulatory overall effect on immunity and orchestrate their activity through diverse cytokine secretions or by direct cell-cell contact. The CD8⁺ cytotoxic T lymphocytes are effector cells that secrete and respond to cytokines that can target infected cells and induce programmed cell death (Kimata & Yoshida, 1994). The T cell immunological activity and response can be monitored by assessing the CD4⁺/CD8⁺ ratios that illustrate critical T cell functions (Kimata and Yoshida, 1994). Previous *in vitro* studies have shown that GH effects on normal and neoplastic human T cells enhanced proliferation is mediated by local increase expression of IGF-I (Geffner, et al., 1990; Merchav, Tatarsky, & Hochberg, 1988; Mercola, Cline, & Golde, 1981; Mosmann & Coffman, 1989).

The CD4⁺ T lymphocytes demonstrate the production of a large number of cytokines known as Type 1 cytokines with IL2 and IFN-gamma being the main cytokines secreted. Enhanced activity of Th2 type cells evokes a strong antibody responses and relatively weak cellular activity (Cherwinski, Schumacher, Brown, & Mosmann, 1987; Mosmann & Coffman, 1989). Th2 cells produce cytokines known as Th2 cytokines and include IL4, IL6, IL10 and TNF- α . As previously mentioned, long-term therapy with rhGH administration in GHD children effectively reduces TNF- α level in these patients (Andiran & Yordam, 2007).

Importantly, IL-10 is well known for having essential immunoregulatory functions with direct influence on the activity of several leukocytes subsets. Its potent anti-inflammatory properties, suppress the expression of inflammatory cytokines such as TNF- α , IL-6 and IL-1 from activated pro-inflammatory macrophages (Donnelly et al., 1999). The IL10 induction of anti-inflammatory cytokines has not yet been explored in healthy individuals that have used rhGH.

As rhGH is currently used by elite and recreational athletes, it is important to further investigate the cellular effects of rhGH on the immune function of healthy males. The purposes of this study were therefore, to evaluate and monitor the immunological effects of seven days short-term administration of rhGH on: NK cell activity, number, phenotype subsets; the T cell numbers (CD4⁺ and CD8⁺) and production of cytokines IL2, IL4, IL6, IL10, TNF- α and IFN- γ : and finally the CD4⁺/CD8⁺ ratio.

Methods

Thirty healthy males were randomly assigned to either a treatment (rhGH, n=15, 24.5 \pm 6.5 yrs) or Placebo (P, n=15; 26.1 \pm 6.6 yrs) group. Daily injections of either rhGH (1mg) or placebo (0.9% sodium chloride) were administered intramuscularly during a period of seven days. Prior to each injection all participants received a standard breakfast (protein shake) followed by 15 min rest. Blood samples (EDTA and Heparin tubes) were collected via puncture of the antecubital vein by a qualified phlebotomist at baseline (day 0) and at days 8, 15, 22 and 29 after 7 consecutive days of injections.

All participants were required to read and sign an informed consent form that was approved by Bond University Human Research Committee. Exclusion criteria included: (i) regular use of therapeutic and/or recreational drugs, (ii) contraindications or risks to exercise (recent injuries/risk factors for maximal strength testing). Sports Medicine Australia pre-exercise

screening tool was used to filter out people at high risk for certain exercise related complications. The criterion for selection was based on the American College of Sports Medicine's (ACSM) guidelines (2000) for pre-exercise screening and testing.

Blood Analyses

The immunological analysis involved the assessment of NK cell numbers, function and phenotypes, CD4⁺/CD8⁺ numbers and further assessment examined cytokine production by Th1 and Th2 cells – (IL-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)- α and interferon (IFN)- γ). All methods were performed using previously described methods (Marshall-Gradisnik, et al., 2008). Whole blood was collected into either heparinised or EDTA blood collection tubes and PBMC were isolated from using Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Determination of the lymphocyte phenotypic subsets (CD4⁺/CD8⁺) was performed using IMK lymphocyte test kit (Becton Dickinson Immunocytometry Systems, California, USA) as previously described (Marshall-Gradisnik, et al., 2008). List mode parameters were collected for 10,000 cells within the lymphocyte gate and positive staining was calculated based on the subsets control specimens. T cells were defined as CD4⁺CD3⁺T cells (T suppressor cells), CD3⁺CD8⁺ T cells (T helper cells), B cells as CD19⁺B cells, and NK cells as CD3⁻cells, (CD16⁺ and/or CD56⁺). Flow-cytometry analyses were carried out using a FACSCalibur (Becton Dickinson) as described (Mozaffari, et al., 2004). Lymphocytes populations were identified by forward and side-scatter analyses.

The NK lymphocyte cytotoxicity was assessed as previously described (Marshall-Gradisnik, et al., 2008). Briefly, PBMCs were isolated from whole blood using ficoll-Hypaque (GE Healthcare) through density gradient centrifugation. The NK cells were labelled with 0.4% PKH-26 (Sigma, St Louis, MO) and resuspended at a final concentration of 5×10^6 cells/mL.

The target cells, K562 cell line were used at a concentration of 1×10^5 cells/mL. The K562 cells were cultured with NK cells in RPMI-1640 culture media (Invitrogen, Carlsbad, CA) for 4 hours in 37°C incubator with 5% CO₂, at a ratio of 25:1. Following incubation, cells were stained with Annexin V-FITC conjugated mAB and 7-AAD reagent (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Percentage of cell death was measured via flow cytometry as previously described (Donnelly et al., 1999).

Quantification of NK cells phenotype and NK cells were isolated from whole blood according to manufacturer's instructions using RosetteSep Human Natural Killer cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC) and Ficoll-Hypaque density centrifugation. Samples were then labelled with mAB CD56-FITC (BD Bioscience, San Jose, CA) and CD16-PE (BD Bioscience, San Jose, CA) according to manufacturer's specifications and analysed using flow cytometry.

The Th1 and Th2 profiles were assessed in whole blood samples 10mL of blood were collected into lithium heparin tubes from all participants (BD Bioscience) and layered over Ficoll-Hypaque for isolation of white blood cells (WBC) following centrifugation at 400g for 40 minutes and washed twice and resuspended in RPMI-1640 culture medium containing 5% foetal bovine serum (FBS). Isolated CD4⁺T cells were mitogenically stimulated with or without 1µg of phytohemagglutinin (PHA). Cells were cultured in a 96 well plate at a concentration of 1×10^6 cells/mL at 37°C with 5% CO₂ for 72 hours. Supernatants were then removed from cells and assessed for Th1, Th2 cytokines using cytometric bead array kit (BD Bioscience) as specified by the manufacturer.

Double antibody radioimmunoassay system (RIA) was applied for quantitative *in vitro* diagnostic measurement of IGF-I within serum using a commercial RIA (IGF-I kit – Bioclone – Australia). Briefly, IGF-I was separated from its plasmatic binding protein then,

the analyte competes with ^{125}I labelled tracer antibody for binding to a constant amount of antibody. A second antibody coupled to magnetisable polystyrene particles was used to separate antibody bound from free ^{125}I labelled tracer antibody as specified by the manufacturer.

Data Analyses

The statistical analyses were performed using SPSS software (version 17.0). General Linear Model with repeated measures was applied and criterion for significant difference was set at $p \leq 0.05$ with Bonferroni post hoc correction applied.

Results

Both groups were not significantly different ($p > 0.05$) in height (P; 178.9 ± 5.9 cm, rhGH; 178.8 ± 9.5 cm), weight (P; 83.1 ± 9.1 kg, rhGH; 81.2 ± 17.9 kg) or age (P; $n=15$; 26.1 ± 6.6 yrs; rhGH 1mg/day ; $n=15$; 24.5 ± 6.5 yrs).

The flow cytometry results revealed no significant differences ($p > 0.05$) between or within groups for NK cell numbers, phenotype or NK activity from baseline (day 0) to day 29 (Table. 1).

Table 1. Natural killer cells absolute numbers (Mean \pm SEM), lysis and phenotype in MF(AU) from day 0 to day 29 for P (placebo) and rhGH groups.

Assay	Group	Day0	Day8	Day15	Day22	Day29
NK number	P	669 \pm 325.1	587.8 \pm 222	611.4 \pm 322.8	555.5 \pm 348.3	550.7 \pm 270.4
	rhGH	560.4 \pm 328	492.5 \pm 229.7	492.8 \pm 190.3	474.79 \pm 160.9	535.2 \pm 154
Nk lysis	P	35.30 \pm 4.57	41.96 \pm 6.58	67.80 \pm 3.87	31.42 \pm 4.76	40.80 \pm 6.61
	rhGH	34.85 \pm 7.63	33.91 \pm 5.97	61.64 \pm 3.17	41.18 \pm 5.73	44.23 \pm 7.23

Nk phenotype	P Bright	217.8±162	224.8±67	257±72	197±161	203.3±104
	rhGH Bright	313.8±158	402.3±345	302.1±221	281.3±86	242.1±140
	P Dim	5.606±2011	5.428±1091	5.788±866	4.391±2653	4.860±1429
	rhGH Dim	5.687±1245	5.309±1827	5.072±2032	4.937±1175	5.210±1034

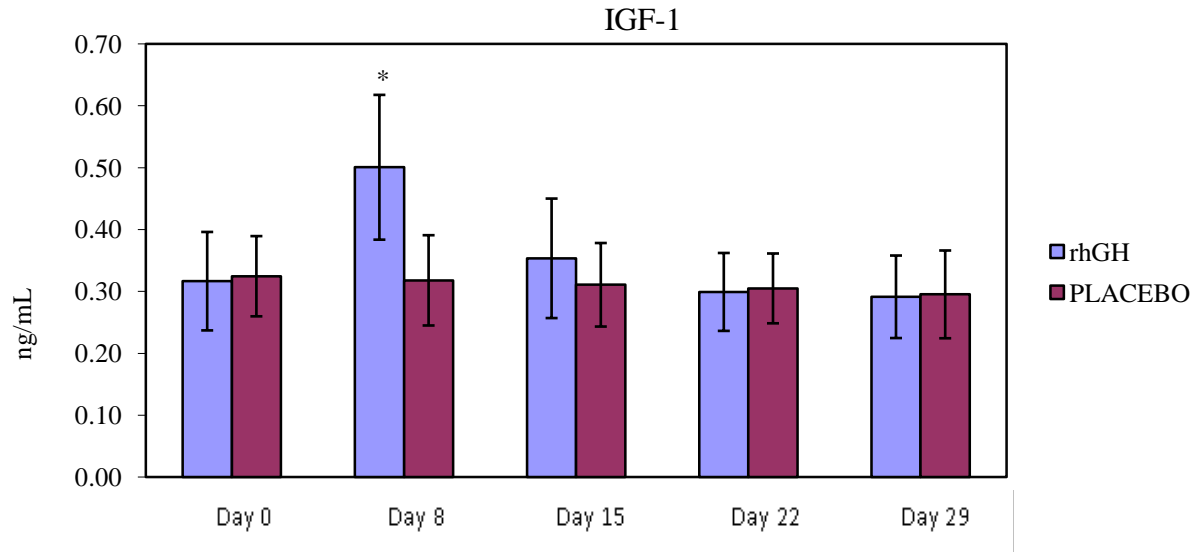
The T cells number (CD4⁺ and CD8⁺), CD4⁺/CD8⁺ ratio and cytokyne production by TH1 and TH2 were also analysed by flow cytometry and showed no significant differences between or within groups for CD4 and CD8 numbers from Day 0 (P= 3700.08±315.52 mean fluorescence) or for CD4/CD8 ratio from day 0 (rhGH=3751.00±297.7 and P=1.35±0.40; rhGH=1.26±0.43) to Day 29 (P=3765.42±293.5; rhGH=3830.2±149.63 and P= 1.55±0.47; rhGH=1.28±0.44 mean channel fluorescence).

The T cell cytokyne production of IL10 increased significantly in rhGH group from day 15 (P=46.32±44.16; rhGH=30.16±23.09 mean channel fluorescence) to day 22 (P=74.28±36.10; rhGH=65.32 ± 35.37 mean channel fluorescence) and remained increased at day 29 (P= 107.50±60.92; rhGH=57.21±54.57 mean fluorescence) as shown in Figure 2. In contrast, IL2, IL4, IL6, TNF- α and IFN- γ showed no significant ($p \geq 0.05$) difference from baseline to day 29 in both groups (Table 2.).

Table 2. T cell cytokine production from baseline to day 29 for placebo (P) and rhGH groups in MF(AU).

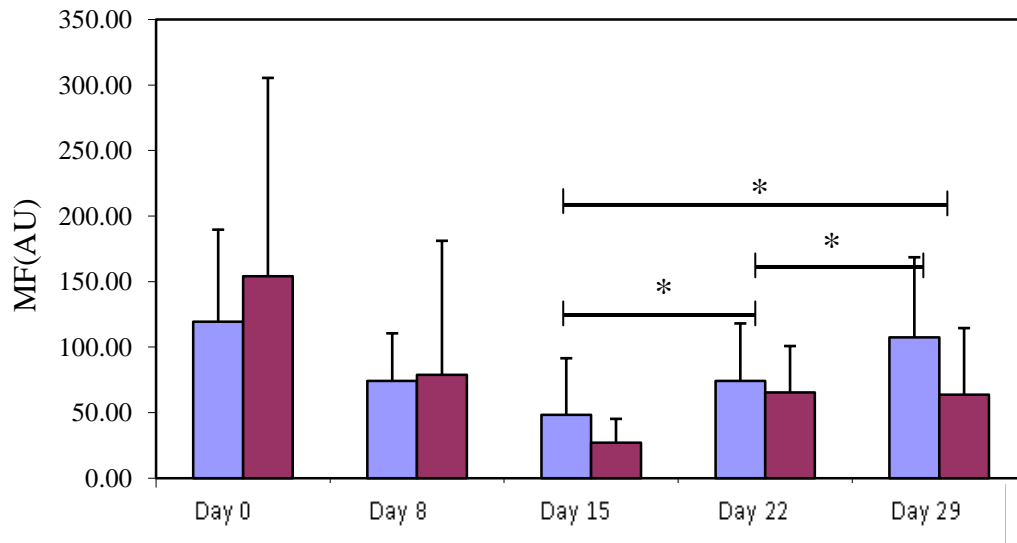
Assay	Group	Day0	Day8u	Day15	Day22	Day29
IL2	P	427.77±195.76	501.30±201.52	374.60±144.90	401.76±192.02	519.94±181.65
	rhGH	517.02±115.57	345.29±172.65	355.30±202.78	464.27±217.19	499.13±153.21
IL4	P	8.29±3.22	7.47±2.36	8.93±3.79	8.05±1.76	12.42±5.97
	rhGH	10.08±3.83	7.08±2.20	8.67±6.39	8.60±5.65	13.69±9.67
IL6	P	4796±1608	4193±1814	2635±1666	2188±1733	3730±1941
	rhGH	5289±1862	3243±1512	3122±1478	3670±2025	3584±1651
TNF-α	P	156.70±60.78	138.33±37.80	135.90±96.87	102.65±42.18	125.73±52.39
	rhGH	141.81±46.94	180.33±73.80	131.64±59.42	152.22±72.23	131.02±54.61
IFN-γ	P	481.73±175.98	373.19±213.82	368.45±250.25	338.02±150.64	430.83±254.47
	rhGH	453.15±258.86	382.38±236.18	382.77±155.94	294.37±161.92	296.51±161.27

A significant difference ($p \leq 0.01$) was found in serum IGF-I concentration in the rhGH group at day 8 (0.49 nmol/mL) compared to baseline (0.32 nmol/mL) (Figure 1.) while no other time point was significant for this group. There was no significant difference in serum IGF-I for the control group from baseline to day 29.



* Significant difference between day 8 and days 0, 15, 22 and 29 for $p \leq 0.05$ in the rhGH group.

Figure 1. IGF-1 analysis in serum per collection day (0 to 29) for groups.



* Significant increase ($p \leq 0.05$) from day 15 to days 22 and 29 in IL10 secretion by the rhGH group.

Figure 2. IL10 secretion from T cells from baseline to day 29 of the study.

Discussion

The aims of this study were to evaluate in young males, the immunomodulatory effects of short-term administration of rhGH on NK cell number, activity and phenotype, T cell cytokine production of IL2, IL4, IL6, IL10, TNF- α and IFN- γ and CD4⁺/CD8⁺ ratio in human PBMCs, with particular attention to the possible correlation to IGF-I production.

The present study indicated no changes in NK cell count, phenotype or activity in either the experimental or placebo group. In contrast, in experiments with 24 month old Wistar rats, rhGH replacement therapy (2 mg/kg daily for 10 weeks) significantly improved several lymphocyte functions such as NK cell activity (Baeza, et al., 2008). Interestingly, a previous study confirmed that both total NK cell count and particularly CD16⁺ NK cell subtype were reduced in GHD patients compared to controls (Sneppen et al., 2002). However, the studies presenting significant results on NK cell were performed for a period of at least ten weeks, suggesting the time course of GH replacement may be a key factor.

In addition, serum IGF-I level significantly increased on day 8 within the rhGH group and along with the high numbers of IGF-I receptors known to be on NK cells (Kooijman, et al., 1992), it was predicted that NK cells activity would be altered after short-term administration of rhGH. However, rhGH induce IGF-I release did not alter NK cells activity nor their number or phenotype in our study. Serum IGF-I concentration in the rhGH group and Placebo group showed only a weak correlation with NK cell activity.

The current results indicate that IGF-I has the potential to exert anti-inflammatory actions through stimulation of IL10 production in CD4⁺ T lymphocytes. The IL-10 cells may inhibit the production of Th1 cytokines (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001) leading to the prevalence of Th2 cells. IGF-I may induce a shift from the production of Th1 to Th2 cytokines, leading to a diminution of cellular immune responses and a stimulation of

antibody-mediated responses. It would appear that rhGH for short-period of time may exert its stimulation effects of Th2 through IL 10 release in healthy population with possible anti-inflammatory action.

The rhGH therapy is a recognised procedure for enhancing immune function in GH deficient populations (Crist et al., 1987; Sneppen et al., 2002; Serri et al., 1999; Andiran and Yordam, 2007), and our data suggest that a dose of 1mg/day for seven days may potentiate immune function of healthy young males through the significant increase in the release of IL10 by CD4⁺ T lymphocytes.

Conclusion

The rhGH has been used by athletes with the intention of improvement of performance. Moreover, there is no evidence of its effect on the immune function. In this paper, it was shown that rhGH stimulated IL10 secretion by CD4⁺ T cells in healthy young males.

Although the lymphocyte numbers between both groups were not significantly different from baseline to day 29, cytokine production showed a non significant decrease followed by a significant increase from day 15 to day 22 and 29 and from day 22 to day 29. In conclusion, cytokine release can be affected by short-term rhGH administration in healthy young males suggesting that rhGH may act on CD4⁺T lymphocytes by increasing IL10 production and may then be a key component in T cell activation.

Conflict of interest statement:

The authors have no conflicts of interest regarding this research.

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