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Full Length Article

Plasmid Based Expression and Bioactivity Evaluation of Caprine Growth Hormone Gene Cloned from a Local Pakistani Goat Breed, Beetal

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Abstract

Growth hormone cDNA of Beetal goat (*Capra hircus*), an indigenous breed of Punjab, Pakistan was amplified by RT PCR and gene including leader sequence was cloned in pTZR57 cloning vector. The cGH-pTZR57 clone was confirmed by restriction digestion and sequence analyses before finally sub-cloning the gene in pND - a mammalian expression vector. The clones were again confirmed by restriction digestion and PCR analyses. Highly purified, super coiled recombinant caprine growth hormone-pND (rcGH-pND) construct was used to transfect Vero cell lines for expression studies. The *in vitro* expression of cGH was determined by dot-ELISA. After confirming its *in vitro* cell line based expression, rcGH-pN, pND constructs and phosphate buffer saline (PBS) were separately injected to 4 weeks old balb/c female mice intramuscularly. Total forty five animals were used to determine the biological activity and randomly divided in to three groups with fifteen mice in each group. Five animals from each group were used to monitor the *in vivo* biological activity by evaluating the body weight gain and tibia epiphyseal width assays from zero week up to four weeks with one week gap. Significant increase ($P<0.05$) gain in body weight and in tibia epiphyseal width was observed in animals inoculated with rcGH-pN. Thus it is concluded that recombinant plasmid of cGH cDNA may be used as supplement to increase the meat production. The effects of the same plasmid on milk production may be checked in future experiments using Beetal goat. © 2014 Friends Science Publishers

Keywords: Caprine growth hormone; Mammalian expression vector pND; Vero cell line; Beetal growth hormone

Introduction

The economy of Pakistan is mainly dependent upon agriculture and livestock is the second important sector of agriculture. Pakistan is rich in its livestock, however, due to urbanization and increasing population size, demands for meat and milk is also increasing. Since past, efforts are being made to cope with these demands by different conventional methods such as selection breeding, improved management, establishment of more dairy herds and utilization of various scientific techniques (Zhang *et al.*, 2013). One of these methods is to strength existing and/or create new infrastructure to facilitate the milk supply along with the stress on milk production (Chattha *et al.*, 2013). Beside all these conventional methods, one of the latest scientific techniques is the use of recombinant technology for the production of valuable proteins like bovine growth hormone (bGH). It is a multifunctional naturally occurring non-glycosylated protein hormone secreted from the anterior pituitary gland having 190 amino acids. Its biological effects are broadly classified as either somatogenic or metabolic which results in linear growth and milk production of the ruminant system (Pell and Bates, 1987). The somatogenic effects are mediated by insulin like growth factor-1 (IGF-1) while the metabolic effect involves a variety of tissues and general metabolism of carbohydrate, lipid, protein, and minerals essential for growth (Etherton and Bauman, 1998). For different biological roles and commercial application in animal husbandry, GH has been cloned from a number of species and expressed in various expression systems like prokaryotic (Wingfield *et al*., 1987; Mukhopadhyay and Sahni, 2002; Khan *et al.*, 2007, Khalid *et al.*, 2008) and eukaryotic system (Hawkins and Nakamura, 1999) to produce high levels of recombinant protein, which is being commercially used for enhancement of milk and meat production in cattle (Baldi, 1999; Bauman, 1999).

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Because of high genetic potentials and low productivity of milk and meat in most of South East Asian cattle and small ruminant breeds, the exogenous administration of recombinant homologous GH approaches on one hand provide the key for boosting the productivity of ruminants to increase the food output (meat or milk) per unit of food source input. This is because of reduction in animal waste products and expenditures for animal feed production (Bauman, 1992). On the other hand, the state of the art plasmid based expression technology is getting popular in recent years (Patil *et al*., 2005). For example, to screen the targets identified from genomic projects, to shuffle molecules for vaccination or direct *in vivo* production of hormones and other therapeutics or preventive applications (Draghia-Akli *et al.*, 2006). This economically feasible gene delivery technology and plasmid mediated gene transfer is also emerging as an excellent candidate for agriculture applications to optimize production and animal welfare (Draghia-Akli and Fiorotto, 2004; Cunningham, 2005).

Goats (Capra hircus) are fastest growing ruminants in Pakistan and about 25 goat breeds are in the country and two wild relative such as Markhor and Ibex (Khan *et al.*, 2008; Shahzad *et al.*, 2012). These are important for poor livestock farmers to make a living and according to recent data over 64 million goats are available in Pakistan (GOP, 2013). Some species are good for meat or milk production. For example, Beetal goats are famous for its milk and meat production and is called poor man's cow (Iqbal *et al.*, 2008; Qudus *et al.*, 2013). These goats are found in almost all irrigated areas of the Punjab including districts of Jhelum, Gujarat, Mandi-Bahaudddin, Sialkot, Gujranwala, Lahore, Sheikhupura, Faisalabad, Sargodha, Jhang, Multan, Sahiwal and Okara (www.haasil-foundation.com/).

Keeping in view the importance of Beetal breed's contribution towards meat and milk production, this study was designed to clone the caprine growth hormone (cGH) cDNA as a supplement agent into a mammalian expression vector, to carry out expression studies *in vitro* using animal cell line and then to evaluate its biological response *in vivo using* animal model. This may help to develop feasible plasmid based delivery system for growth hormone cloned from various farming animals to enhance the production of milk and meat in high demand market like South East Asian countries and help to raise livestock sector.

Materials and Methods

Plasmid Preparations

Caprine GH-pTZR57 construct in DH5α cells with accession # DQ 307368 by Khan *et al.* (2005) was available in Biotechnology Laboratory, Department of Biochemistry, PMAS-Arid Agriculture University Rawalpindi. The clone was refreshed from its glycerol stock and plasmid DNA was extracted by the method described by Maniatis *et al.* (1989). Similarly, mammalian expression vector pND cloned in DH5α cells was also refreshed by streaking and growing on Lauria-Bertani (LB) media prepared by the method mentioned by Maniatis *et al.* (1989). Plasmid DNA of pND vector was extracted to be used in sub-cloning experiment. This vector contains constitutive immediate early promoter and enhancer sequences from cytomegalovirus (CMV) and 1000 bp intron sequence for optimal gene expression under *in vitro* and *in vivo* conditions. Before starting experiments, the protocols were reviewed and approved by the ethical committee of PMAS Arid Agriculture University Rawalpindi.

Sub-cloning of cGH in pND

The cGH-pTZR57 construct was restricted with *Xba I* and *Bam HI* to produce the 692 bp long cGH gene with leader sequence. Similarly pND vector was modified by restriction digestion with *Nhe I* and *Bam HI* enzymes. The fragments were purified from gel by gel extraction kit (Fermentas, USA) and purified products were ligated with T4 ligase using standard ligation procedure provided by Supplier (Roche, USA). The ligation mixture was then transformed into chemically competent B10 strain of *E. coli*. The positive clones were confirmed by restriction digestion using EcoRV and agarose gel electrophoresis.

Expression Studies

DNA used for expression studies was prepared by Quantum prep™ Plasmid Midiprep kit (Bio-Rad, USA). DNA concentration was determined by spectrophotometer at optical density of 260 nm and 280 nm. The ratio of 260/280 nm was used to determine the quality of DNA. Most of DNA samples gave ratio between 1.7 and 2.0. The samples with lower ratio were retreated with chloroform-isoamylalcohol to remove proteins. The revived Vero cells obtained from National Institute of Health (NIH), Islamabad were used for expression studies. The cells were grown in Medium 199-Hepes containing 10% fetal bovine serum, 1% penicillin streptomycin solution (PSS), 1% amphotericin B and 0.6% L-glutamine by culturing the cells in 12 wells plate (Corning, USA) and incubated in the humidified CO_2 incubator at 37°C. Cells with 80% of confluence were used for transfection experiments using calcium phosphate co-precipitation method described by Wigler *et al.* (1978). After transfection, the cells were incubated from 48 to 96 h. Thereafter the media and cell monolayer were harvested and analyzed by dot blot.

Bioactivity Evaluation

The biological activity of the construct i.e. rcGH cDNA was evaluated in white BALB/c, an albino female mice by weight gain and tibia epiphyseal width assays using endotoxin free plasmid DNAs (Greenspan *et al.*, 1949; Zhu *et al.*, 1997). Forty five, four weeks old BALB/c mice were divided into three groups containing fifteen animals each

and kept at animal house facility of National Institute of Health (NIH), Islamabad to acclimatize for one week before inoculating with plasmid DNAs. First group of mice were injected with 100 µg pND vector without insert in100 µL of phosphate buffer saline (PBS), second group was injected with 100 µL of PBS solution and third group was injected with 100 µg rcGH-pND in 100 µL PBS of intramuscularly. All animals were examined for change in body weight and width of the tibial epiphysis starting from zero week till the end of four weeks with one week interval. The data was analyzed by using analysis of variance (ANOVA) through MS Excel Data analysis tool Version 2007. The means with significant difference were compared with Duncan Multiple Range Test (DMRT) using M-STAT C.

Results

The primary objective of this study was to evaluate the bioactivity of cloned GH cDNA from caprine breed; Beetal using novel DNA-based expression technology.

Sub-cloning of cGH cDNA in pND Vector

The sub cloning of cGH cDNA in pND vector was confirmed by restricting the clone with restriction enzyme; *EcoR V* which generated an expected fragment of 692 bp. The obtained result is shown in Fig. 1.

Expression Studies

Expression of cGH cDNA was done in Vero cell line. The maximum level of expression was observed with 2.5 µg of construct i.e., rcGH-pND at 72 h of post transfection in cultural media. The pND without insert i.e., rcGH was used as negative control, while commercially available GH hormone was used as positive control in these *in vitro* expression studies, which showed no expression and strong expression, respectively (Fig. 2). Similarly level of expression, determined in cell lysate, showed no detectable expression at any incubation hours with both concentrations of rcGH-pND used i.e., 2.5 µg and 5 µg (Fig. 3).

Activity Evaluation

The bioactivity evaluation of plasmid based recombinant caprine growth hormone (rcGH-pND) by weight gain and tibia width bioassays in BALB/c female mice showed that there was significant increase in the weight ($P<0.05$) as well as in width of tibia ($P< 0.05$) of the mice from zero to fourth week of the experiment showing gradual normal growth pattern in comparison to PBS and pND control (Table 1, 2).

Discussion

Expression studies carried out by other investigators using GH cDNA from other species like human and ovine (which is identical to caprine and thus can be used in both animals; ovine and caprine) using different cell lines i.e., COS-7, Verots S3, SP2/0 respectively showed the presence of recombinant GH protein in cultured supernatant of transfected cells (Min *et al.*, 2009). The positive results of our expression studies in animal cell line model have provided the bases to proceed for determining the biological activity of rcGH-pND in animal model. Thus, the results of weight gain and tibia width assays showed that rcGH-pND was biologically active in the muscle of animal and may be used as supplement to increase the biomass and meat production in goat breeds as well as in sheep breeds since

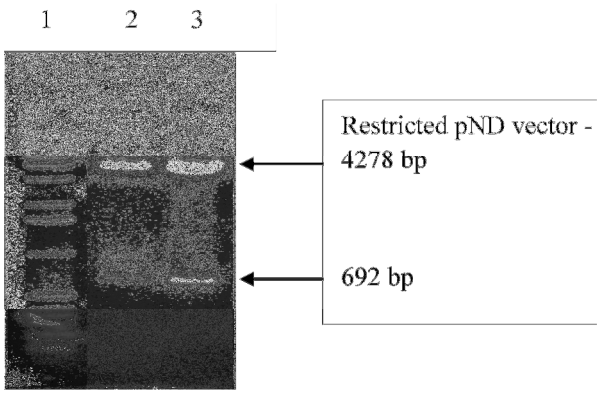

Fig. 1: Restriction analysis of rcGH sub-cloned in pND mammalian vector. Lane 1. 1 Kb ladder (Invitrogen, USA), Lane 2-3. rcGH cDNA of ~692 bp obtained from pND constructs restricted with *Eco* RV

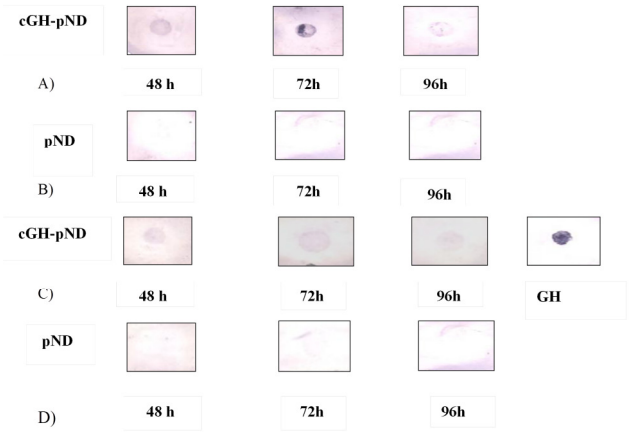

Fig. 2: Confirmation of expression of rcGH-pND contruct from cell's culture media by dot blot. (A) The Vero cells transfected with 2.5 µg of pND having rcGH incubated from 48 h to 96 h. (B) The Vero cells transfected with 2.5 µg of pND vector incubated from 48 h to 96 h. (C) The Vero cells transfected with 5 µg of pND having rcGH from 48 h to 96 h. GH is commercially available growth hormone, used as a positive control. (D) The Vero cells transfected with 5 µg of pND vector incubated from 48 h to 96 h

Table 1: Weight gain data of BALB/c mice from 1-4 weeks post inoculation of recombinant caprine growth hormone (rcGH) in mammalian pND vector

Treatment	0 week	1st week	2nd week	3rd week	4th week
pND	20.5 ± 2.12^{eA}	23.0 ± 1.41^{dA}	26.5 ± 0.71^{cA}	30.5 ± 0.71^{bB}	34.5 ± 0.71^{aB}
PBS	19.5 ± 0.71^{eA}	23.5 ± 0.71^{dA}	26.5 ± 2.12^{cA}	31.0 ± 1.41^{bB}	33.5 ± 3.54^{aB}
rcGH-pND	16.5 ± 0.71^{eB}	19.5 ± 0.71^{dB}	28.0 ± 1.41^{cA}	34.0 ± 1.41^{bA}	36.5 ± 2.12^{aA}

Table 2: Tibia width data of BALB/c mice from 1-4 weeks post inoculation of recombinant caprine growth hormone (rcGH) in mammalian pND vector

Treatment	0 week	1st week	2nd week	3rd week	4th week
pND	5.28 ± 0.97^{cA}	7.36 ± 1.40^{bA}	9.69 ± 0.05^{bB}	13.98 ± 0.11^{aB}	14.28 ± 0.31^{aB}
PBS	5.38 ± 1.40^{dA}	7.76 ± 1.40^{cA}	9.49 ± 0.81^{bB}	13.67 ± 0.02^{aC}	14.12 ± 0.54^{aB}
rcGH-pND	4.27 ± 0.15^{eA}	6.65 ± 0.15^{dA}	9.03 ± 0.21^{cB}	15.20 ± 0.21^{bA}	15.69 ± 0.16^{aA}

Means with similar smaller superscripts in rows are insignificant (P>0.05) with each other. Means with similar capital superscripts in columns are insignificant (P>0.05) with each other

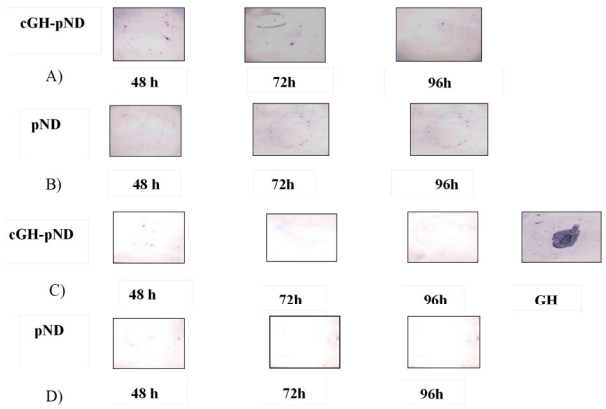

Fig. 3: Confirmation of expression of rcGH-pND contruct from cell lysate by dot blot. (A) The Vero cells transfected with 2.5 µg of pND having rcGH incubated from 48 h to 96 h. (B) The Vero cells transfected with 2.5 µg of pND vector incubated from 48 h to 96 h. (C) The Vero cells transfected with 5 µg of pND having rcGH incubated from 48 h to 96 h. GH is commercially available growth hormone, used as a positive control. (D) The Vero cells transfected with 5 µg of pND vector incubated from 48 h to 96 h

caprine growth hormone is identical to ovine (Ascacio-Martinez and Barrera-Saldaña, 2012). However, effect of plasmid based rcGH for enhancement of milk production needs to be determined. The weight gain and tibia width assays has been used in a number of studies especially to evaluate the effect of human growth hormone in dwarf little mice (Bellini and Bartolini, 1993), recombinant human growth hormone (George *et al.*, 2007; Kwak *et al.*, 2009), recombinant DNA derived human growth hormone/somatotropin in hypophysectomized rats (Zhu *et al.*, 1997). The reason being that recombinant human growth hormone is used to treat growth hormone deficiency in children and adults and wasting in AIDS patients (Cox *et al.*, 2007).

Draghia-Akli *et al.* (2003; 2006) also used plasmid based technology to deliver growth hormone releasing hormone (GHRH), a releasing hormone for growth hormone, to various animal species for screening, toxicology and therapy. A GHRH-expressing plasmid was engineered for efficient expression in skeletal muscle and following intramuscular injection enhanced by electroporation in piglets, it was observed that GHRH is synthesized in injected muscle, secreted into blood stream and stimulated normal pituitary GH production and release. The release GH then in turn stimulated the growth of piglets. In another study carried out by Meng *et al.* (2004) observed enhanced growth in sheep when plasmid expression vector having sheep GHRH was injected in muscle tissue of the animals. These studies indirectly support present study in which plasmid DNA of growth hormone rather than plasmid DNA of GHRH has been used to evaluate the biological activity. Thus all these studies (indirect) including ours (direct and first of its kind) support the notion that plasmid based gene delivery system is more feasible in terms of administrating plasmid rather than administrating the product of gene in purified form which is not cost effective. However, further studies are required to determine the effect of plasmid-based delivery of GH rather than protein based delivery on milk production by farming animals.

In conclusion, the recombinant plasmid based technology seems to be a good source of gene delivery and *in vivo* transgene expressions. Further, it is cost effective therapy in comparison to exogenous administration of recombinant homologous GH especially in resource limited setting to enhance the genetic potential of animals to increase meat and milk production.

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