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In vitro Suppression of Prolactin During Later Stages of Egg Lay in Domestic Hen (*Gallus gallus Domesticus*) Anterior Pituitcytes by RNA Interference

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Abstract: Prolactin (PRL) is a peptide hormone secreted by anterior pituitary gland. PRL increases as per the age and reproductive cycle of birds from normal physiological levels to extremely higher level there by affecting the ovulation, egg formation, oviposition, egg production and hyperprolactinemia. This is more pronounced and persistent after 72 weeks of age in birds. Hence a study was conducted in anterior pituitary primary cultured cells obtained from 72 and 82 weeks old white leg horn (WLH) hens to knock down the PRL gene expression by siRNA and observing its effects on PRL, PRL mRNA, protein content of PRL, prolactin receptor (PRLR) and Growth Hormone (GH) mRNA to unravel the functional role of PRL at 72 and 82 week age by siRNA for PRL. Three siRNAs were designed as per standard siRNA protocols and studied the suppression of PRL gene expression in primary cultured cells procured from adult chicken anterior pituitary glands in *in vitro* conditions. Average percentage reduction of PRL in anterior pituitary primary cell culture following siRNA transfection was 82 and 60% at 72 and 82 weeks respectively. Protein content of PRL was significantly ($p<0.01$) decreased in siRNA transfected cells compared to controls. Growth Hormone (GH) mRNA and PRL receptor (PRLR) mRNA levels did not change significantly ($p<0.01$) between control and treated cells. Results clearly suggested that the siRNA designed for PRL specifically decreased PRL gene expression in *in vitro* conditions. Level of PRLR mRNA and GH mRNA levels expression did not follow the similar pattern of PRL gene expression in anterior pituitary cells. It is concluded that, construction of short specific siRNA for PRL significantly decreased PRL, PRL mRNA and protein content of PRL without showing any effect on PRLR and GH between the two age groups of birds. These results may lead to construction of short specific siRNA for stable and chronic suppression of PRL gene expression during embryogenesis before an increase of PRL occurs for long term knock down of PRL in *in vivo* conditions. In conclusion, understanding of hyperprolactinemia and the involvement of PRL may provide the basis for the development of therapeutic drugs or methods against hyperprolactinemia by RNA interference.

Key words: Hyperprolactinemia, PRL, PRLR, GH, in adult chicken anterior pituitary cells, siRNA

INTRODUCTION

RNA interference (RNAi) is a sequence-specific gene silencing process that occurs at the posttranscriptional level. Prolactin (PRL) is a peptide hormone mainly synthesized in the anterior pituitary gland and is involved in biological actions in vertebrates. In avian species, PRL is associated with broodiness (Bedecarrats *et al*., 1999), frequent pauses between the sequences of egg lay with concomitant decrease in egg production during the active period of egg lay (Reddy *et al*., 2001, 2002) in hen. This phenomenon is more pronounced and persistent in native breed of birds (Reddy *et al*., 2006a). Elevated levels of PRL are associated with increased nesting frequency (>90% of the day), incubation of eggs, antagonistic to gonadotrophic and gonadal hormones, delayed sexual maturity, gonadal involution, delayed ovulation and egg formation, more inter sequence pause days, anorexia, dipsia (<5% of normal intake), changes in intermediary metabolism and hyperprolactinemia, there by affecting the reproductive performance in hen (Reddy *et al*., 2003, 2002). Control of higher levels of PRL to physiological ranges reverse the above with increased egg production without any deleterious effects on other physiological processes, as evidenced by our earlier findings such as by using antiprolactin agent (2-alpha-bromoergocriptine) through feed (Reddy *et al*., 2001)., active immunization studies against PRL(Reddy *et al*., 2006b, 2007) and PRL releasing factor i.e., Vasoactive Intestinal Peptide (VIP) (Reddy *et al*., 2008).These are all short term goals with many disadvantages for practical application. To overcome these disadvantages, RNAi approach is being taken in this study as it has recently come as a revolutionary breakthrough in unraveling the gene function. During ontogenesis, PRL plays a role on homeostasis (Al-Kahtane *et al*., 2005) and the control of

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osmoregulation (Bedecarrats *et al*., 1999). In turkey ontogenesis, PRL and PRL mRNA increases from 5 days before hatching in the anterior pituitary gland. Circulating level of PRL increases from 2 days before hatching (Bedecarrats *et al*., 1999). Thereafter, these levels reach a plateau between the day of hatch and 3 days of age (Bedecarrats *et al*., 1999, Gen Hiyama *et al*., 2010a). In this context, further study is essential to unravel the functions of PRL during later stages of egg lay in domestic hen. In order to identify the functional role of PRL during late embryogenesis, an *in vitro* study was conducted by Gen Hiyama *et al*. (2010b), to suppress the PRL transcription and translation in turkey embryonic anterior pituicytes by RNA interference. It was observed that, siRNA for PRL significantly suppressed the PRL gene, PRL mRNA, protein content of PRL without having any effects on expression profiles of PRLR and GH in turkey embryonic pituicytes (Gen Hiyama *et al*., 2010c, 2008). However, further studies are required in *in vivo* condition to test the efficacy of PRL suppression and its effects on other physiological parameters. However, some breed of birds show decline in egg production around 12 to 18 months of age showing irregular egg production cycles to complete cessation of egg lay for the remaining two thirds of their life cycle. Since only one ovary is functional in avian species and several of the white follicles undergo atresia throughout the life span (Reddy *et al*., 2002, 2008, 2006b and 2007) which needs to be investigated by RNAi. Hence there is an immediate need to increase egg lay with available resources under similar managemental practices to combat the growing demand of the ever increasing human population. Due to increasing urbanization, changing lifestyle the per capita consumption of eggs has increased tremendously hence an increasing egg production becomes pivotal particularly in rural areas from native breed of hens. During the last decade or so poultry research focused to improve reproductive efficiency through various interventions viz. chemical and immunological interventions as well as modified managemental practices. Attempts were made by our team by chemical interventions using an antiPRL agent and active immunization methods to PRL and Vasoactive Intestinal Peptide (VIP) to augment egg lay (Reddy *et al*., 2001, 2003, 2005, 2006b) by minimizing the inter sequence pause days between the sequences of egg lay in hen so as to increase clutch length (Reddy *et al*., 2010) in chicken up to 72 weeks of age. With recent invention of RNAi, it is possible to extend the egg lay beyond 72 weeks of age with the available resources under similar managemental practices by knock down PRL specifically and to bring down the PRL to normal level to suppress the negative effect of PRL on gonadotrophic hormones to improve egg formation, ovulation and egg lay in hen without any deleterious effects on other physiological processes by RNA interference. This may provide the basis for understanding of the role of PRL during broodiness in hen and turkeys. Broodiness is more persistent in turkeys and native bred of birds. Understanding the physiological mechanism of broodiness and the involvement of PRL may provide the basis for the development of new therapeutic procedures or drugs against this by using RNA interference in vivo by RNAi expression vector without disrupting the other roles of PRL in normal physiological processes in galliformes.

MATERIALS AND METHODS

Designed three siRNA's for PRL based on chicken and turkey PRL mRNA using RNAi software. Anterior pituitary glands were collected from White leghorn chicken during later stages of egg lay at 72 and 82 weeks of age (n = 60). Anterior pituitary cells were isolated and cultured in the primary cell culture as described by Gen Hiyama *et al*. (2010c). From all the groups, anterior pituicytes were seeded to a 12 well plates containing a mixture of 40 nM of siRNA, Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen) followed by incubation of cells without antibiotics for 18 h at 37°C in humidified 95 air-5% CO_2. The culture medium was replaced with DMEM containing chicken serum and ITS. (Gen Hiyama *et al*., 2010c). After four hours, cells was stimulated by adding VIP. Total RNA was extracted by trizol method from cultured cells. The first strand cDNA was synthesized from RNA with the use of random primers. PRL mRNA levels, PRL receptor (PRLR) and Growth Hormone (GH) were estimated by quantitative real time PCR after reverse transcription. We designed the primer sets based on the cDNA sequence of PRL. The suppressive effects of siRNA on the protein level were assessed. Total protein was extracted from cultured cells and subjected to western blotting followed by incubation with PRL polyclonal antibody and alkaline phosphatase labeled anti-rabbit IgG antibody. Immunoreactive signals were detected by exposure to x-ray film (Gen Hiyama *et al*., 2010c).

RESULTS AND DISCUSSION

Birds were sacrificed as per institute animal ethics committee recommendations at 72 and 82 weeks of age and anterior pituitaries were isolated, cultured and conducted the siRNA for PRL in anterior pituitary primary cultured cells. Significant suppression of PRL mRNA expression was observed with all three siRNA's designed. The best siRNA was selected in this experiment based on the highest knockdown of PRL. Twenty four hours after the introduction of siRNA, 82 and 60% reduction in PRL was observed in siRNA transfected pituitary cells obtained from 72 and 82 week aged birds respectively (Fig. 1). However, the suppressive effects of RNA interference was prominent in the cells derived from 72 weeks of age compared to

Fig. 1(a-f): PRL expression in control and siRNA transfected anterior pituicytes with VIP stimulation(5×10-7M) and without VIP stimulation in primary cultured cells of anterior pituitary glands obtained from at 72 and 82 weeks of age in domestic hen. PRL gene was clearly suppressed (C and F) following RNA interference. (A): Control (b): siRNA with VIP (c): siRNA without VIP (d): Control (e): siRNA with VIP and(f): siRNAwithout VIP

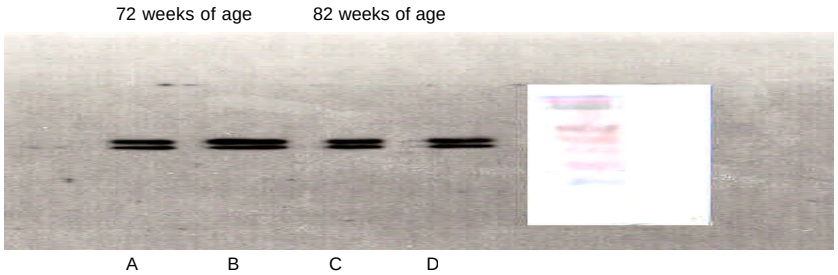

Fig. 2(a-d): Effect of siRNA for PRL on GH expression between 72 and 82 weeks of age in hen anterior pituicytes. (siRNA of PRL do not show any effect on GH expression). (a): control (b): siRNA of PRL transfected cells, (c): Control and (d): siRNA of PRL transfected cells

82 weeks of age. The reasons for the differences in suppressive efficacy of siRNA between 72-82 weeks of age are unknown but may be related to circulatory and pituitary concentration of PRL between 72 and 82 weeks of age. In controls, PRL mRNA levels were significantly ($p<0.01$) higher in 72 weeks of age as against 82 weeks of aged pituicytes. This confirmed that the knock down of PRL is directly related to its quantity in anterior pituicytes as per the reproductive cycle in domestic hen (Reddy *et al*., 2002). PRL secretion profiles in plasma and anterior pituicytes decreases as the age of hen advances. This may be attributed to significantly ($p<0.01$) higher knock down of PRL by selected siRNA in anterior pituicytes at 72 weeks of age due to presence of significantly higher levels of PRL, compared to 82 weeks of aged pituicytes. It is quite interesting to observe that the secretory pattern of the pituicytes decreases after 72 weeks of age. Reasons to this are unknown. With this observation, the basal levels of PRL mRNA were almost 25% fold higher in the anterior pituitary cells obtained from 72 weeks of age compared 82 week old anterior pituicytes. This may give some clues that the PRL secretion rates will vary as per the age, efficiency of designed siRNA and its transfection in *in vitro* conditions. This may also depend on the dose of sRNA treatment/protocol required to suppress the expression of PRL during the later stages of egg lay. In the cultured cells addition of VIP did not increase the levels of PRL mRNA. The level of PRLR and GH mRNA were also assessed. It did not show any significant difference on PRLR and GH mRNA by siRNA transfection. This is in conformity with the results obtained from Gen Hiyama *et al*. (2010c) (Fig. 2). These results suggest that the effect of siRNA was specific to PRL transcripts but not to the level of PRLR mRNA. Significant reduction in PRL was observed in the siRNA transfected anterior pituicytes cells obtained from 72 week age old cells by dot blot analysis. The expression level of PRL was enhanced by VIP stimulation in siRNA transfected cells, as against the levels in controls without VIP treatment. These results suggested a correlated between PRL and protein content of PRL in siRNA treated cells with VIP stimulation. In controls, there was no significant increase in PRL level was observed by VIP stimulation. Our results are inconformity with the results of Gen Hiyama *et al*. (2010c) which may be attributed to the saturation of immunoreactive signals in controls with a pattern of PRL level PRL mRNA (Gen Hiyama *et al*., 2010c). VIP treatment accelerates the PRL gene expression by modulation of cAMP, protein kinase A, protein kinase C, Ca^{2+} and other second messenger such as c-fos (Kansaku *et al*., 1998; Al-Kahtane *et al*., 2005; Gen Hiyama *et al*., 2010c, 2008). Reason for the lack of responsiveness of PRL to VIP treatment is unknown, may be related to the above mentioned or may be due to other reasons which require further study on the VIP-PRL pathway under the current cell culture conditions as suggested by Gen Hiyama *et al*. (2010c). It is concluded that, PRL and PRL protein content were specifically knocked down by siRNA in *in vitro*

conditions. For long term suppression of PRL in *in vivo*, a stable and chronic suppression of PRL is required by using either lentiviral constructs or a nonpathogenic E.Coli that can be introduced during embryogenesis before an increase of PRL occurs in embryonated chicken eggs for long term benefits to enhance egg lay in turkey, hen and various other avian species to control the menace of broodiness in the near future. However, outcome of our findings may leads to control of excessive levels of PRL to normal levels by using RNAi science.

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