Original Article
Physiology

Mechanistic Investigations of the Dual Activity of Gonadotropins Using Target Tissue cAMP Level as a Response Parameter

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ABSTRACT [ENGLISH/ANGLAIS]

Gonadotropins like human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) exhibit dual activities on target tissues. The status of cAMP as a second messenger in these activities has been examined experimentally. Direct correlation with target tissue cAMP concentration, has been observed in the case of thyrotropic effect of hCG on thyroid tissue and the ovarian ascorbic acid depletion effect of hCG on superovulated immature rat ovaries. Marked difference in the response of buffalo tissues in comparison to rat tissues has been observed. No correlation could be observed between cAMP levels and ovarian ascorbic acid content in the case of PMSG action. Surprisingly a peptide derived from buffalo Prolactin appears to have anti-gonadotropic effect as far as ovarian ascorbic acid content is concerned.

Keywords: Hormones, Gonadotropins, cAMP, ELISA

RÉSUMÉ [FRANÇAIS/FRENCH]

Les gonadotrophines comme la gonadotrophine chorionique humaine (hCG) et sérum de jument gravide gonadotrophine (PMSG) présentent des activités duales sur les tissus cibles. Le statut de l'AMPc comme second messager dans ces activités a été examiné expérimentalement. Corrélation directe avec la concentration de l'AMPc tissu cible, a été observée dans le cas d'effet thyréotrope de l'hCG sur le tissu de la thyroïde et l'appauvrissement de la couche d'acide ascorbique de l'ovaire effet de l'hCG sur superovulées ovaires de rats immatures. Différence marquée dans la réponse des tissus buffles en comparaison avec les tissus des rats a été observée. Aucune corrélation n'a pu être observée entre les taux d'AMPc et la teneur en acide ascorbique de l'ovaire dans le cas de PMSG action. Étonnamment un peptide dérivé de buffle prolactine semble avoir antigonadotrope effet dans la mesure où la teneur en acide ascorbique de l'ovaire est concerné.

Mots-clés: Hormones, gonadotrophines, AMPc, ELISA

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INTRODUCTION

Gonadotropins are a group of protein hormones secreted by the anterior pituitary gland of all vertebrates as well as by placental cells of primates and equids. Gonadotropins stimulate gonads primarily but are known to affect non-gonadal tissues as well. For example, the thyrotropic activity of human chorionic gonadotropin (hCG) has been reported [1]. HCG and LH share structural similarities with TSH and the definitive role of hCG and LH as thyroid stimulators has been postulated [2]. Pregnant mare serum gonadotropin (PMSG) also exhibits dual activity but in a different sense. While it is predominantly Luteinizing hormone like in horse and mare, it has both LH-like and FSH-like activities in heterologous species like rats and mice (Nikki kumari and Muralidhar- unpublished work). Gonadotropins and thyrotropin are reported to use cAMP as a second messenger in their action [3]. The role of cAMP in the mediation of LH action was first demonstrated by Marsh *et al.* [4], LH increases the tissue level of cAMP in bovine corpus luteum slices [5], in whole ovaries from mouse [6] and pre-pubertal rat [7], in isolated rat ovarian follicles [7] and in porcine granulose cells [8]. In addition to this, LH also appears to stimulate steroidogenesis in the same tissues. The work of K Ahren *et al.* [9] showed that cAMP level tend to increase in case of whole pre-pubertal ovary,

isolated follicles from 32 day old rats injected with PMSG(on day 30) and also in isolated corpora lutea from 33-39 day old rats injected with PMSG(on day 30).

In light of this available information, it was of interest to know whether any correlation can be observed between cAMP levels (if it is truly a second messenger) and these reported activities like follitropic activity (FSH-like), thyrotropic activity (TSH-like) and ascorbic acid depleting activity (LH-like) existing in the same hormone like hCG or PMSG.

MATERIALS AND METHODS

Immature female Holtzman strain rats were obtained from the departmental animal house facility. Animals were maintained in 12L: 12D in air conditioned rooms at 22 to 260C, food (pellet diet) and water were supplied ad libitum. Pituitary glands, thyroid glands and ovaries of buffaloes were procured from a local abattoir. Pituitary glands were from animals of two different age groups (old and young).bovine LH β antiserum was a kind gift from J G Pierce (Formerly of Department of Biological Chemistry UCLA, USA). Ovine TSH β antiserum and cAMP antiserum were purchased from NHPP, Dr A F Parlow.Concanavalin A, methyl α-D-mannopyranoside, ascorbic acid, hCG, PMSG, Theophylline, *o*-phenylene diamine (OPD), 4 chloro alpha napthol, phenyl methane sulphonic acid (PMSF) were purchased from Sigma-Aldrich, St Louis USA. ELISA plates were purchased from Greiner, Greiner Bioscience Germany. 2, 6-dichlorophenol indophenol (DCPIP) was purchased from Loba Chemie, Bombay. Goat anti rabbit IgG-HRP conjugate and medium range SDS PAGE molecular weight markers were purchased from Bangalore-Genei Ltd India. All other chemicals used were of analytical grade.

Estimation of protein was done according to the method given by Lowry *et al* [10]. SDS-PAGE was performed according to the method given by Laemmli [11].

Electro transfer of the protein bands from the polyacrylamide gel was done according to the method suggested by Towbin *et al* [12].

Competitive ELISA

Direct binding ELISA was done according to standard procedures [18]. In the case of competitive ELISA, the competitor was prepared in acetate buffer and 100 µL of it was added to the wells first. This was followed by the addition of primary antibody. In the case of competitive ELISA, B_0 wells were always set where instead of the competitor 100 µL of the buffer was added. % binding was calculated as

Percentage binding= B-NSB/B_0-NSBX100

Where B = wells with competitor

B_0 = wells without competitor

Estimation of Ascorbic Acid

A standard calibration curve was constructed using 0- 80 µg of ascorbic acid in 2.5mL metaphosphoric acid–citrate solution. 2.5mL of the dye solution was delivered quickly into the tube mixed well and the absorbance was read at 540nm at 20^{th} ± 1 second after the addition of the dye in the spectrocolorimeter. Ovarian ascorbic acid was estimated essentially as per the assay of Parlow [13].

In vitro estimation of cyclic AMP in ovary of rats and buffalo after induction with hCG and bu LH

The ovaries from both sides were taken out of experimental rats and weighed in the nearest mg balance and kept in a ice-chilled Petri plate containing KRBG+ theophylline. Subsequent steps were carried out in ice. The ovaries were minced with the help of a blade and a slide. 100 µl of the minced tissue was aliquoted per tube. Three separate groups were made, one was taken as the control; one of the rest two was tested to see the effect of hCG and the other one to see the effect of bu LH. To the control group 20 µl of KRBG + theophylline was added and to the test groups, different hormone doses are added. Then the micro centrifuge tubes were incubated at 37°c in water bath for 20 min. The reaction was terminated by keeping the micro centrifuge tubes in boiling water bath for 3 to 5 min. Extraction of cAMP was done by adding 0.1 N of HCl. Then all the samples were brought to neutral pH through the addition of 1 N NaOH. The samples are then ready for estimation of cAMP through competitive ELISA

Competitive ELISA for Estimation of cAMP

In the case of competitive ELISA, at first the micro-titre plates were coated with the cAMP conjugated with thyroglobulin. Then the wells were blocked with 1% gelatin in 10mM PB (pH 7) varying concentrations of the competitor (cAMP or test samples) were prepared in 0.1M Sodium acetate buffer pH 4.75 containing 0.1% gelatin and 100 µL of it was added to the wells first, followed by the addition of primary antibody. 100 µL of the buffer, instead of sample, was added to B_0 wells. After 3 h of incubation secondary antibody was added to the wells and incubated for 1 h. Substrate was added to all the

wells. As soon as the color development occurred, the reaction was stopped with the addition of 1 M oxalic acid and the absorbance was recorded at 490nM in an ECIL ELISA plate reader. Net percentage binding was plotted against concentration of cAMP taken. Values were read in the cAMP standard curve by intrapolation.

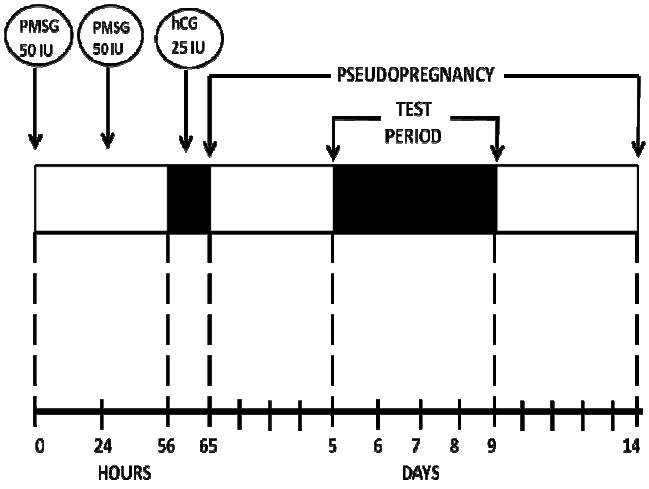
All measurements were done in triplicate experiments and results are shown as mean and standard deviation unless otherwise stated.

RESULTS AND DISCUSSION

In vivo and In vitro Bioassays

One of the well-known bioassays for LH measures the depletion in ovarian ascorbic acid content developed by Parlow [13].

Figure 1: This figure shows model system employed for the study of LH action on ovarian ascorbic acid (Adapted from [13])



In this model (Fig 1) super-ovulated immature female rats of 35 days age are given test dose of hCG (30IU, 60IU, 90IU) and four hours later the animals are killed by cervical dislocation. Ovaries and thyroids are taken out and the surrounding blood vessels and adhering fat tissue are cleaned. The left ovary was used for estimation of ascorbic acid while the right ovary was utilized for cyclic AMP estimation. The calibration curve for ascorbic acid estimation showed that the method works in the range of 0 to 60 µg of AA (Fig 2). A dose dependent depletion in ascorbic acid content was noticed (Fig 3).

Figure 3 also indicates that a synthetic peptide derived from buffalo prolactin (PRL) internal amino acid sequence, when administered to these superovulated immature rats in the same model, caused an increase in ovarian ascorbic acid content. It might indicate that this PRL derived peptide can reverse the effect of LH but this has to be tested further. It was earlier been shown that exogenous PRL inhibits release of LHRH in lactating rats *in vivo* [14]. Hence it can be said that PRL probably has inhibitory effect on LH action in ovarian ascorbic acid depletion. But the pathway of action is still not deciphered.

Figure 2: This figure shows standard curve for the estimation of ascorbic acid using the dye 2,6, dichlorophenol indophenol. Equal volumes of the ascorbic acid solution in metaphosphoric acid and the dye solution were mixed and the absorbance was measured at 20th sec thereafter at 540 nm in a spectrocolorimeter. X axis denotes the concentration of ascorbic acid and the Y axis signifies optical density at 540 nm. Data represents average of two experiments.

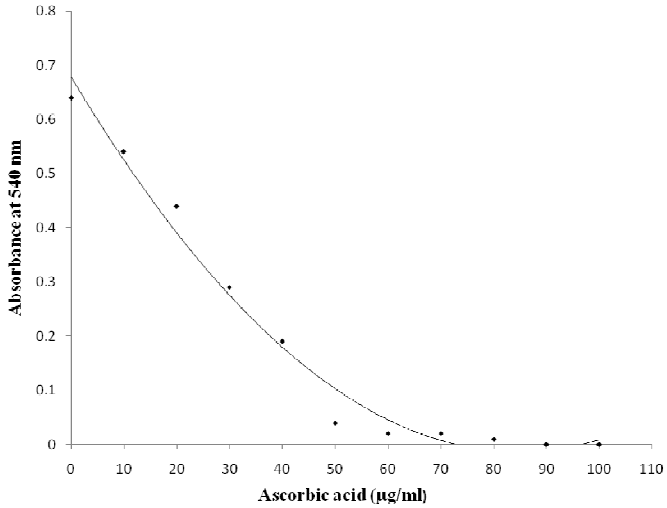
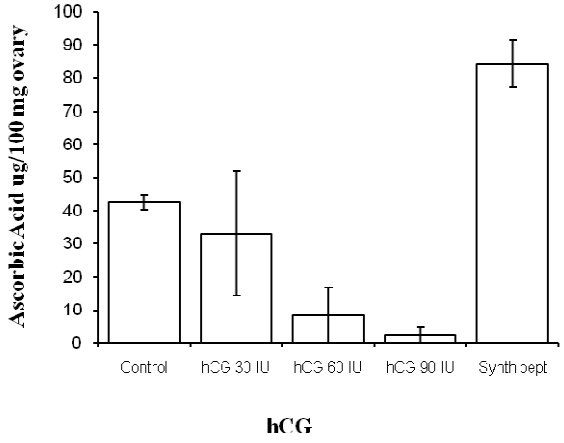


Figure 3: This figure shows effect of hCG and a PRL derived synthetic peptide on ovarian ascorbic acid content in superovulated immature rat.



Another established fact is that gonadotropins act on their target tissues by generation of the second messenger

cyclic AMP by stimulating adenylate cyclase [1]. This has been confirmed (Table 2). It was also observed that this effect of LH was cycloheximide insensitive (data not shown). Therefore induced or constitutive protein synthesis is not required for this effect of LH on ovarian ascorbic acid. Earlier work had shown that the administration of LH to these rats results in increase in ovarian content poly A rich RNA [15] and that this effect could be observed within 30 minutes of LH administration. Hence the effect of LH on rat ovary appear to be both metabolic (non-genetic) and developmental (genetic).The mediation of LH effect on ovarian ascorbic acid by cAMP was confirmed with additional observations. LH (i.e. hCG) was observed to increase ovarian cAMP content .In this model the left ovary was used for ascorbic acid estimation while the right ovary was used for cAMP estimation. A calibration curve was obtained with standard cAMP. This showed an ED_{50} of 4 picomoles (Fig 4).

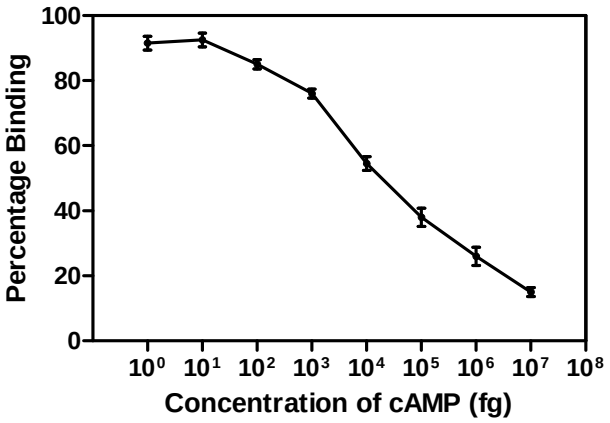
Table 2: This table shows ascorbic acid ascorbic acid depletion by LH

Experiment No#	Group	Dose (µg/rat)	Change in Ascorbic Acid content from control value
I	Control	-	77.11 ± 9.5*
II	LH	3.2	32.71± 4.5*
III	dbcAMP	50	66.77± 8.5*

* *P < 0.005 when compared with the corresponding control animals (d.f. = 9)*

Cyclic AMP was extracted from the right ovarian tissues of the superovulated immature rats using 0.1 N HCL. Later the samples were neutralized. Like ascorbic acid depletion cAMP levels were stimulated by hCG in a dose dependent manner. 30 IU of hCG could increase the cAMP content 1.43 fold over that of control animals. The 60 IU hCG administered group had 2.76 times higher cAMP content than the control and the content increased to 12.14 fold in the 90 IU group (Fig 4). Thus hCG is able to stimulate cAMP levels in super-ovulated rat ovaries i*n vivo.* What is surprising in these observations is that cAMP content was measured 4 hours after the administration of the hormone and still the levels of cAMP were high. All previous studies have measured cAMP within 10 minutes after stimulation by LH (hCG) [9]. The turn-over of ovarian ascorbic acid and cAMP may not be same. It is for this reason that the percent depletion in ovarian AA content and percent increase in ovarian cAMP content do not match at any given dose of hCG (LH) as seen from Fig 3 and 5. The question of mediation of a hormone by cAMP can be tested at various levels. One, whether hormone administration results in increase in cAMP; two, whether adenylate cyclase is stimulated; three, whether cAMP phosphodiesterase is inhibited; four, whether cAMP or dbcAMP brings about the same effect; five, whether inhibition of phosphodiesterase results in hormone action; and six, whether stimulation of phosphodiesterase inhibits hormone action. Here the first condition/prerequisite only has been fulfilled for establishing the second messenger status of cAMP in LH action on ovary with regard to ascorbic acid depletion.

Figure 4: This figure shows calibration curve for estimation of cAMP



Further, to check the effect of first and second dose of PMSG and the final dose of hCG individually during the process of super-ovulation induction and ascorbic acid depletion in immature female rat ovaries another experiment was performed. The ovaries were checked for the ascorbic acid content after each injection. With respect to the control animals the ovarian ascorbic acid content in the PMSG and hCG treated animals are significantly high (Fig 6) confirming the role of ascorbic acid in the ovulation. Which subsequently proved the role of ascorbic acid in facilitating luteal steroidogenesis. It is interesting to note that in this particular action, PMSG is functioning as FSH as it stimulates ovarian growth. Previous work from our laboratory had demonstrated that in the process of ovarian growth, PMSG (or FSH) action results in increased cAMP levels in the ovary [16]. All cellular constituents like glycogen were found to increase. Hence the increase in cAMP levels in the tissue

has to be interpreted with caution. cAMP has been unambiguously shown to decrease glycogen by promoting glycogenolysis. Hence parallel increase in cAMP as well as glycogen is difficult to explain even though direct correlation was observed.

Figure 5: This figure shows dose dependent effect of hCG on cAMP content of ovaries from super-ovulated immature rats *in-vivo*.

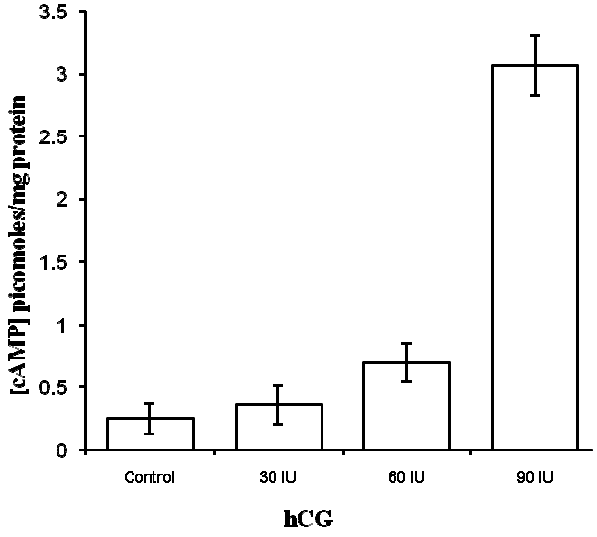
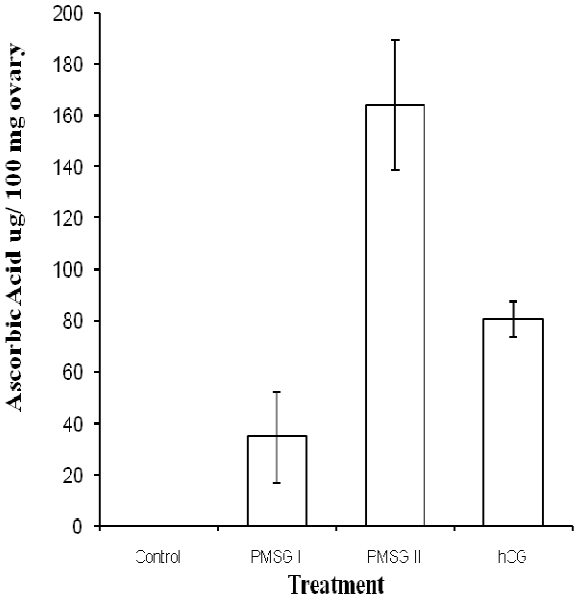


Figure 6: This figure shows ascorbic acid content of ovary after each of PMSG and hCG injections



In vitro Assay of cAMP from Ovarian Tissues in Respect to their cAMP Content

It was of interest to see whether the *in vivo* effect of LH (hCG) could be reproduced *in vitro*.

In this case ovaries from mature rats were taken out and placed in Krebs-Ringer-Bicarbonate buffer pH 7.4 and containing glucose (11 mM) and theophylline (tp) (8mM). The ovaries were minced. The ovarian minces from the control group were incubated with 20 µL of KRB buffer containing glucose and theophylline for 20 minutes.

Ovarian minces from the experimental animals were exposed *in vitro* to different doses of hCG and purified buLH (Fig 7) and buLH (Fig 8) stimulated cAMP production in the minced ovarian tissues *in vitro*.

As can be seen 2 IU of hCG *in vitro* caused a 100 fold increase in ovarian cAMP content over control (0.033 pico moles of cAMP). Whereas the group which was incubated with 4 IU of hCG showed a 160 fold increase in cAMP content, 8 IU hCG could cause a 328 fold increase in cAMP.

Figure 7: This figure shows *in vitro* effect of different concentrations of hCG on rat ovarian cAMP content.

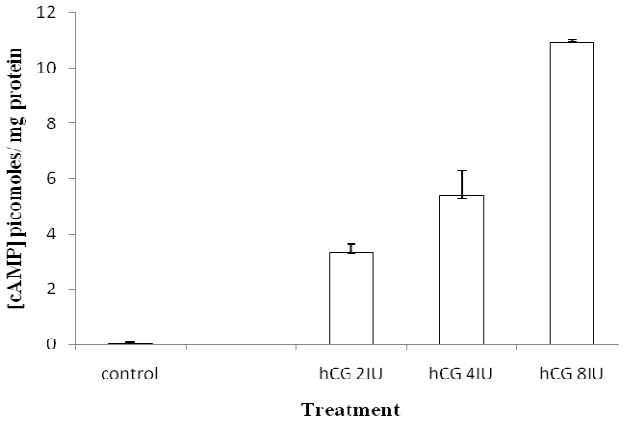
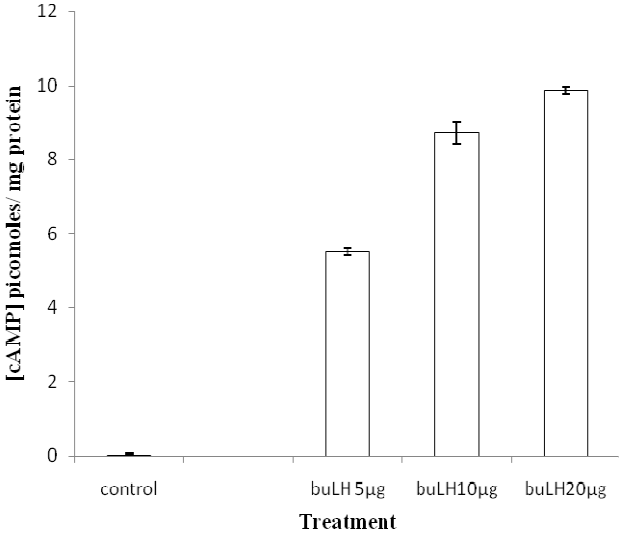


Figure 8: This figure shows *in vitro* effect of buLH on rat ovarian cAMP



The same samples were also used to elucidate the effect of purified buLH. The purified buffalo LH could elicit a dose dependent response. The three doses of buLH namely 5, 10, 20 µg / 20 µL were observed to cause an increase of ovarian cAMP levels 165.4 fold, 262 fold, 296 fold respectively over the controls (Figure 8).These preliminary results indicated that buffalo LH has a potency of 800 IU/ mg protein but *in vitro*. In the classical ventral prostate assay or the ovarian ascorbic acid depletion assay bovine LH was reported to possess 3000 IU/ mg protein compared to 12000 IU / mg protein in hCG. This is puzzling as *in vivo* it should have much less bio potency than *in vitro* as the hormone is cleared from blood very fast. Sialylated proteins (e.g. hCG) serve longer in plasma than non sialylated proteins (e.g. buffalo LH). More work has to be done in this regard, obviously. It was then decided to work on homologous system.

Figure 9: This figure shows effect of hCG *in vitro* on buffalo ovarian cAMP content.

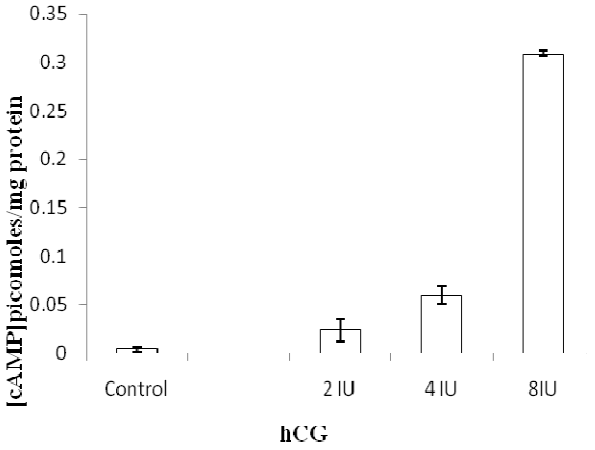
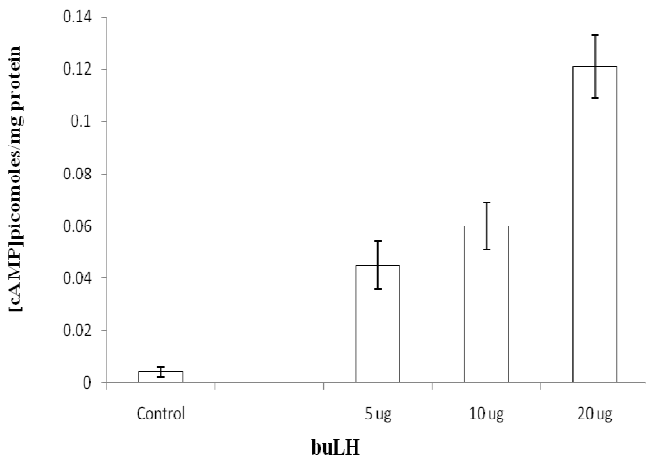


Figure 10: This figure shows *in vitro* effect of buLH on buffalo ovary



Results obtained show that both hCG (Fig 7) and buLH (Fig 8) caused increase in ovarian cAMP content in a dose dependent manner. From these in vitro studies it can be inferred that cAMP indeed plays a decisive role in the mechanism of action of LH/hCG. As the *in vitro* incubation time of ovaries with LH is 20 minutes to relate it with any metabolic effect of LH is really difficult as most of the metabolic effects take time to occur except the ovarian blood flow as from earlier studies it is clearly known that the increase in blood flow occur 6 to 120 sec after i.v. injection of the hormone [17]. Hence the functional relationship between the tropic increase in cAMP and other metabolic effect of LH/hCG in ovarian tissues has to be investigated further.

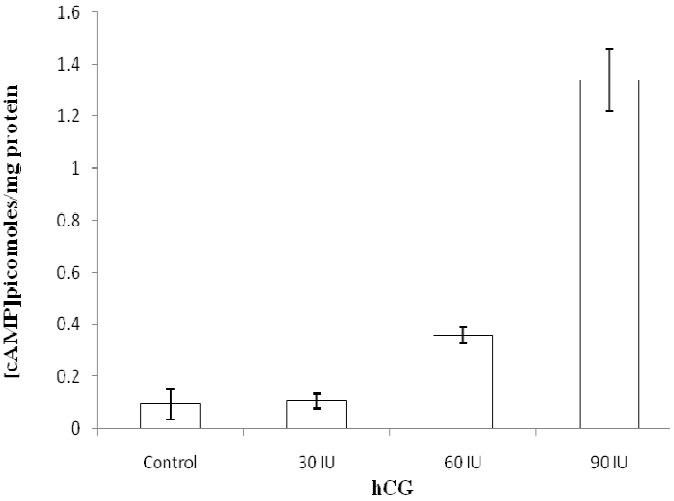
2 IU, 4 IU and 8 IU of hCG caused an increase in cAMP 11.25, 15 and 30.25 fold over the control group. These results show that the buffalo ovary is less sensitive than the other heterologous system (i.e. rat ovary). The response of buffalo ovary to hCG *in vitro* appears to be hardly 10% of the response of rat ovary to the same dose of hCG. Does buffalo have less number of receptors per cell than rat? Does the buffalo ovary has less 'coupling efficiency' between hormone binding and adenylate cyclase activation when compared to rat? More work has to be done to understand this better.

The dose dependent effect of buLH was quiet pronounced as it caused the increasing accumulation of cAMP in the minced ovarian tissues to 6, 15 and 77.25 over basal level of different doses. These results also indicate that the buffalo LH was more active in rat than in homologous buffalo (165, 262 and 296 fold increase in rat compared to 6, 15 and 77 fold increase in buffalo). From the present study it is clear that bu LH was more active in rat (heterologous) than in buffalo (homologous). This could be due to the difference in the developmental state of the ovaries. Further studies are needed to validate the effect of buLH in this *in vitro* bioassay taking in account the different developmental status of the ovaries. However this amounts to 5% at the two lower doses and 20% at the higher dose. This indicates that both the number of receptors and affinity of the receptors in buffalo is less than that in rat. More interesting is the observation that buffalo responds better to buLH than to hCG. This could be the crucial clue to the general failure of Embryo Transfer Technology (ETT) programme in buffalo as everybody has used PMSG/ hCG and not buFSH/ buLH. More work has to be done in this regard.

Thyrotropic Effect of hCG on Rat Thyroid *In-vivo*

Nisula's group [7] demonstrated the effect of hCG in terms of thyroid hormone secretion, iodide uptake, organification and cAMP formation in cultured human thyrocytes. Hence it was decided to check this fact in case of both i*n vivo* and *in vitro* models with respect to change in cAMP content of the target (ovary) and non-target (thyroid) tissues four hours after the doses of hCG were given to the super-ovulated immature rats, the thyroids were removed and the cAMP content was measured therein. Here also, the dose dependent effect of hCG on thyroid cAMP content was observed. A 114.89 % , 382.9% and 1425.5 % increase in cAMP content over the controls were observed at 30, 60, 90 IU hCG respectively (Fig 11).

Figure 11: This figure shows effect of hCG on cAMP content of thyroid.

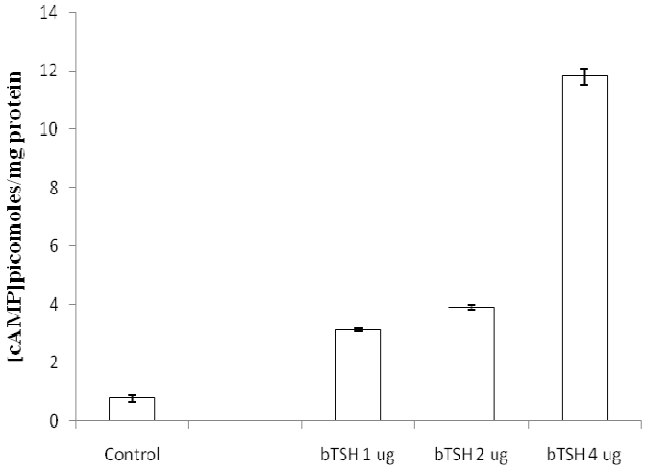


So from all these above data it is clear that hCG has a dose dependent thyrotropic effect as reflected in terms of total cAMP content/mg of protein of the thyroid gland.

Thyrotropic Effect of hCG and buLH on Rat and Buffalo Thyroid *In-vitro*

To validate the results obtained in *in vivo* system, an *in vitro* assay system was developed to confirm the effects obtained with hormones studied. In *in vitro* system, minced rat thyroid glands were incubated with respective doses of hCG. Predetermined volumes of all the samples were incubated with different doses of hCG and bTSH in 20 µL volume. The control group was incubated only with KRBG+T. Result obtained have clearly indicated that bTSH has stimulated thyroid cAMP levels in a dose dependent manner. The buTSH and buLH used in the induction assay of cAMP were our lab preparation and are shown to be free of contamination from each other (data not shown). In comparison to the control values 1 µg of bTSH stimulate cAMP level up to 399 % whereas 2 µg of bTSH caused 497.4% increase in cAMP accumulation in the respective minced thyroid tissues. The effect of 4 µg dose of the bTSH has been found to be more pronounced giving stimulation of the order of 1506.3 % in comparison to the control animals (Fig 12). Biphasic effect of TSH on thyroid follicles is well known but here the effect was observed to be an increase in cAMP content in a dose dependent manner. Hence it can be inferred that threshold level for the bidirectional effect of bTSH is not attained till this dose.

Figure 12: This figure shows effect of different doses of bTSH on cAMP content of rat thyroid in an i*n vitro* assay system.



Under similar conditions, different doses of hCG (2-8 IU/ 20uL) were found to stimulate the cAMP levels. When tissues were incubated with the increasing doses of hCG (2, 4, 8 IU), the cAMP content was found to follow non typical biological response pattern. The highest value of 497.4% increase in cAMP was with 4IU. At 8 IU hCG, there was a decrease in cAMP response which was observed to be 299.7 % (Fig 13). 2 IU hCG caused 197.7 % increase in cAMP content with respect to that of control group. It appears that 4 IU of hCG has activity equal to that in 2µg of bTSH approximately.

Similar to rat thyroids, a biphasic response to hCG. There was approximately 500, 300 and 400 % increase in cAMP in response to 2, 4and 8 IU hCG i*n vitro* (data not shown). In case of buLH the result was quiet significant as it was able to stimulate the cAMP accumulation. Addition of 10 and 20 µg of buLH resulted in accumulation of 1000% and 5000% respectively over controls (Fig 14).

Figure 13: This figure shows thyrotropic effect of hCG on cAMP content of rat thyroid in an *in-vitro* assay system.

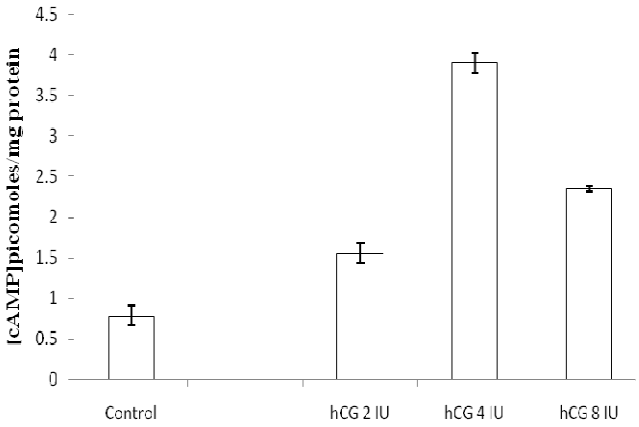
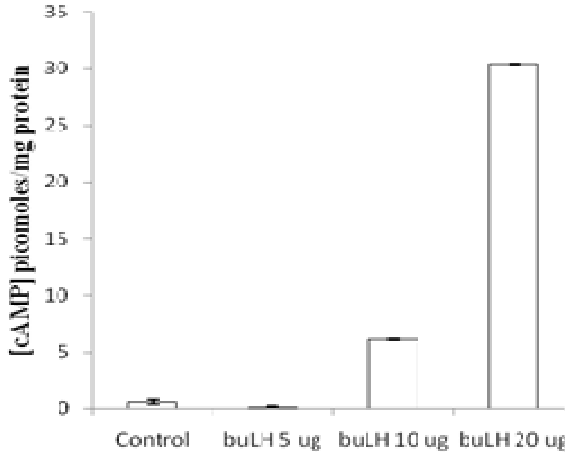


Figure 14: This figure shows effect of buLH on buffalo thyroid cAMP content i*n vitro.*



Hence it can be inferred that hCG and buLH are potent thyroid stimulators acting through cAMP. The only puzzle which eludes understanding based on conventional signaling mechanisms in the present study is the increase in ovarian glycogen caused by FSH [16] and the increase in ovarian ascorbic acid caused by PMSG. Most probably FSH like actions of FSH and PMSG have more than one signaling mechanisms through which they operate in ovaries.

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CONFLICT OF INTEREST

No conflict of interests was declared by authors