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BIOLOGICAL ACTIVITY DETERMINATION OF HUMAN CHORIONIC GONODOTROPIN IN WISTAR RATS

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ABSTRACT

Human chorionic gonodotropin is a hormone produced primarily by syncytiotrophoblastic cells of the placenta during pregnancy.The hormone stimulates the corpus luteum to produce progesterone to maintain the pregnancy.Smaller amounts of hCG are also produced in the pituitary gland, the liver, and the colon. HCG is a chemical created by trophoblast tissue, tissue typically found in early embryos and which will eventually be part of the placenta. Measuring hCG levels can be helpful in identifying a normal pregnancy. Pathologic pregnancy, and can also be useful following an aborted pregnancy. There is also a benefit in measuring hCG in a variety of cancers including choripcarcinoma and extra-uterine malignancies.

KEYWORDS: HCG, FSH.

INTRODUCTION

Human chorionic gonodotropin is a hormone for the maternal recognition of pregnancy produced by trophoblast cells that are surrounding a growing embryo which eventually forms the plancenta after implantation. The presence of hCG is detected in some pregnancy tests. From the time of implantation hCG produced by trophoblast cells take over corpus luteal progesterone production luteinizing hormone (LH), acting on a joint hCG/LH receptor. After that time there are sufficient syncytiotrophoblast cells in the placenta to take over progesterone production from corpus luteal cells. Some cancerous tumors produce this

hormone therefore, elevated levels measured when the patient is not pregnant may lead to cancer diagnosis and if high enough paraneoplastic syndromes however, it is not known whether this production is a carcinogenesis. The pituitary analog of hCG known as LH is produced in the pituitary gland of males and females of all ages. HCG is a glycoprotein composed of 237 amino acids with a molecular mass of 36.7KDa, approximately 14.5αhCG and 22.2KDaβhCG. It is heterodimeric with an α subunit identical to that of luteinizing hormone (LH), follicle stimulating hormone (FSH) thyroid stimulating hormone (TSH), and β subunit that is unique to hCG.

The α(alpha) subunit is 92 amino acids long. The β-subunit of hCG gonodotropin (beta-hCG) contains 145 amino acids, encoded by six highly homologous genes that are arranged in tandem and inverted pairs on chromosome19q13.3-CGB.[1,2,3,5,7,8] It is known that CGB7 has a sequence slightly different from that of the others The two subunits create a small hydrophobic core surrounded by a high surface area to volume ratio:2.8times that of a sphere. The vast majority of the outer amino acids are hydrophobic Beta-hCG is mostly similar to beta-LH, with the exception of a carboxy terminus peptide (beta –CTP) containing four glycosylated serine residues that is responsible for hCG's longer half-life.

Function

Human chorionic gonodotropin interacts with the LHCG receptor of the ovary and promotes the maintenance of the corpus luteum for the maternal recognition of pregnancy at the beginning of pregnancy. This allows the corpus luteum to secrete the hormone progesterone during the first trimester. Progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it can sustain the growing fetus.

It has been hypothesized that hCG may be a placental link for the development of local maternal immunotolerance. For example. hCG-treated endometrial cells induce an increase in Tcell apoptosis (dissolution of T cells). These results suggest that hCG may be a link in the development of peritrophoblastic immune tolerance, and may facilitate the trophoblast invasion, which is known to expedite fetal development in the endometrium. It has also been suggested that hCG levels are linked to the severity of morning sickness or hyperemesis gravidarum in pregnant women.

Because of its similarity to LH, hCG can also be used clinically to induce ovulation in the ovaries as well as testosterone production in the testes. As the most abundant biological

source is in women who are presently pregnant, some organizations collect urine from pregnant women to extract hCG for use in fertility treatment.

Human chorionic gonodotropin also plays a role in cellular differentiation/proliferation and may activate apoptosis.

Production

Naturally, it is produced in the human placenta by the syncytiotrophoblast.

Like any other gonodotropins, it can be extracted from the urine of pregnant women or produced from cultures of genetically modified cells using recombinant DNA technology.

In Pubergen, Pregnyl, Follutein, Profasi, Choragon and Novarel, it is extracted from the urine pregnant women.It Ovidrel, it is produced with recombinant DNA technology.

Human chorionic gonodotropin forms

Three major forms of HCG are produced by humans, with each having distinct physiological roles. These include regular HCG, hyperglycosylated hCG, and the free beta-subunit of hCG. Degradation products of HCG have also been detected, including nicked hCG, hCG missing the c-terminal peptide from the beta- subunit and free alpha-subunit, whichhas no known biological function. Some HCG is also made by the pituitary gland with a pattern of glycosylation that differs from placental forms of HCG.

Regular HCG is the main form of HCG associated with the majority of pregnancy and in non-invasive molar pregnancies.This is produced in the trophoblast cells of the placental tissuie. Hyperglycosylated HCG is the main form of HCG during the implantation phase of pregnancy, with invasive molar pregnancies, and with choriocarcinoma.

Gonadatropin preparations of HCG can be produced for pharmaceutical use from animal or synthetic sources.Some of these are medically justified, whereas others are of a quack nature Humam chorionic gonodotropin can be used as a tumor marker as its β subunit is secreted by some cancers including seminoma, Choriocarcinoma, germ cell tumors, hydatidiform mole, teratoma with elements of choriocarcinoma, and islet cell tumor. For this reason, a positive result in males can be a test for testicular cancer. The normal range for men is between 0-5mlU/Ml. Combined with alpha-fetoprotein, β-HCG is an excellent tumor marker for the monitoring of germ cell tumors.

Hcg and assisted reproduction

Controlled ovarian stimulation (COS) for in vitro fertilization (IVF) is based on various protocols with different ovarian response amongst women with issues of infertility. IVF experts face multiple failed attempts in every day practice, the goal being to try and find the ideal therapeutic approach for each patient. The use of gonadotropins and the variety of expression of its receptors in each patient have been at the top of the research pyramid for many years.

We know by now that LH is involved in follicle maturation, beginning from the antral stage. Primordial and primary preantral are considered gonadotropin independent stages of follicular development, since cumulus and theca cells lack FSH and LH receptors. However, studies have demonstrated the presence of FSH and LH receptors from the secondary preantral and from the antral stage onwards, respectively LH receptors are also present in the theca cells from the secondary preantral stage onwards, while they are lacking FSH receptors. Gonadotropin receptor allocation in follicular cells expresses the two-cell two-gonadotropin theory.According to basic knowledge, LH stimulates androgen production by the theca cells, while FSH promotes aromatase enzyme activity and thus the utilization of androgens as a substrate for estrogen biosynthesis.

FSH regulates recruitment, selection and dominance of ovarian follicles, while LH promotes final maturation and ovulation, making the two gonadotropins act synergistically in the process of follicular growth. Even though the preantral stage can be reached in the absence of LH, it is an important factor in both oocyte and follicular cells development through modification of the steroid and protein micro- and macro-environment. These physiologic changes have a prominent role in oocyte maturation, ovulation and subsequent fertilization and implantation. Studies on non-human primates have shown that LH may act by increasing intra-ovarian androgens, which in turn promote FSH responsiveness in granulosa cells.

hcg is being used in assisted reproduction protocols to mimic the mid-cycle LH surge because of the degree of homology between the two hormones. HCG is characterized by structural similarities with LH, while they share the same receptor, LH/CGR. The factor that distinguishes HCG is the longer half-life of 36 h while the elimination half-life of recombinant LH is estimated to be about 10–12 h. The slower plasma metabolic clearance of hCG consists of a rapid phase in the first 5–9 h following intramuscular (IM) administration and a slower phase in the first 1–1.3 days after administration. Apart from that, hCG

demonstrates a stronger LH/CGR receptor binding affinity, probably due to differences in the carbohydrate moiety, which may make the molecule more sensitive to the binding receptor and is much more potent than LH.

LH and hCG have the same α-subunits and a high cysteine content and also present with an identical natural function; inducing ovulation and supporting lutein cells. Their main differences involve the sequence of the β-subunit, the regulation of the secretion of the two hormones, the carbohydrate component and the pharmacokinetics of clearance of hCG as opposed to LH.

The LH/CGR has an almost ubiquitous distribution in reproductive tissues, a fact that may suggest that the actions of hCG might be more extensive than once thought to be. Although it is mainly located in the gonads, ovary and testis, it can also be found in extra-gonadal reproductive organs, such as the uterus and the fallopian tubes. Moreover, studies have stated that non-reproductive tissues such as the skin, breast, adrenals, thyroid, neural retina and neuroendocrine cells express LH/CGR too. Regardless of the administration or not of FSH, low-dose hCG can support the development and maturation of larger ovarian follicles that have acquired granulosa cell LH/CGRs, possibly providing effective and safer ovulation induction regimens. In a recent study, it was demonstrated that the addition of hCG to rFSH in a short GnRH-agonist protocol throughout the early follicular phase had a beneficial effect in terms of pregnancy rates, while hCG was also associated with better quality embryos. A possible explanation would be the direct induction of theca cells androgen production, which is subsequently transformed to estrogen in granulosa cells through an increased aromatization rate.

In a previous study by our group, it was demonstrated that infertile women pre-treated with hCG seemed to have higher E2 levels on the day of hCG administration for triggering ovulation and resumption of meiotic division, which is related to better quality embryos and increased pregnancy rates.

In a prospective randomized study by Drakakis et al the authors tried to determine whether low dose hCG added to rFSH for ovarian stimulation could produce better results compared to the addition of rLH in women entering IVF-ET in a short protocol, especially in those women with previous failed IVF attempts. Results showed that, due to the use of hCG, less gonadotropin ampoules were needed for ovarian stimulation and higher fertilization and

higher pregnancy rates were recorded. There was also a tendency for a better implantation rate, even in women of advanced reproductive age with higher basal FSH levels, which are often considered to have poor ovarian response to stimulation. In addition, the percentage of mature oocytes and the number and quality of embryos was comparable between rLH and hCG groups. This could state that hCG, in the specific dose and way of administration, had no drawback effect on ovarian stimulation.

A possible explanation provided by the authors could be that the longer plasma half-life of hCG and its greater potency (roughly six to eight times greater than that of LH) leads to highly effective and more stable occupancy of the LH/CG receptors. Given the fact that serum E2 levels in patients who received rLH were significantly lower than in patients treated with hCG, the occupation of the LH/CG receptor in the rLH-administered patients is less than to the hCG stimulated patients. Moreover, it was demonstrated that mRNA levels of the LH/CG receptor after ovarian stimulation were significantly higher among women receiving hCG, compared to those receiving rLH.

Assessing the use of recombinant LH and hCG in oocyte maturation during clinical In Vitro Maturation (IVM), a number of studies have investigated the differences between the effects of these gonadotropins in a well-designed in vitro system. In the absence of the use of FSH, low-dose hCG can support the development and maturation of larger ovarian follicles (≥15 mm in diameter) that have acquired granulosa cell LH/CGRs while, at the same time, it inhibits the demise of smaller follicles lacking these receptors, thus being dependent on FSH stimulation. Dinopoulou et al., studied the effect of recombinant-LH and hCG in the absence of FSH on IVM, fertilization and early embryonic development of mouse germinal vesicle (GV)-stage oocytes. The LH/hCG receptor was expressed in all stages of in vitro matured mouse oocytes and in every stage of early embryonic development. Furthermore, the addition of hCG in IVM cultures of mouse GV oocytes significantly increased the recorded maturation rates.

In search of another potential use of hCG, Filicori et al., studied the possibility of replacing FSH with low-dose hCG in order to complete controlled ovarian stimulation. The authors included infertile patients, separated in two study groups. In the first group, patients received recombinant FSH of hMG throughout COH, while in the second group ovarian priming was induced with recombinant FSH/hMG followed by a low dose of hCG. The authors concluded that low-dose hCG in the late COH stages reduced recombinant FSH/hMG administration

while ICSI outcome had no significant difference. It was also stated that follicle growth and maturation were stimulated regardless of FSH dosage and the protocol led to a reduced number of preovulatory follicles. Moreover, there was no premature luteinization and a more estrogenic intrafollicular environment was recorded in the low-dose hCG group. The same group also tested the effect of hCG on patients with hypogonadotropic hypogonadism. They concluded that the administration of highly purified FSH and low-dose hCG (50 IU/day) led to an acceleration of follicular development in follicles >14 mm and a reduction in the dose of FSH requirement, while the duration of COS was also shortened

Hcg and endometrial synchrony

We have already known for more than two decades that LH/CG receptors are expressed in the human uterus. These receptors are found in multiple cells types, especially in epithelial cells. Based on this knowledge, we can hypothesize that hCG could lead to the improvement of uterine receptivity via the enhancement of endometrial quality and stromal fibroblast function. Moreover, through its actions on insulin-like growth factor binding protein-1 (IGFBP-1) and vascular endothelial growth factor (VEGF), hCG was found to stimulate endometrial angiogenesis and growth, thus extending the implantation window and increasing pregnancy rates. IGFBP-1 is selectively produced by decidualized endometrial stromal cells and represents a marker of decidua formation and its concentrations are cycle-dependent. One study has shown that women investigated before day 10 after the LH surge were IGFBP-1 negative, while all women examined at day 10 or later presented with high intrauterine IGFBP-1 levels. This could serve as a marker that indicates the ending of the window of implantation, leading to the hypothesis that the increase of IGFBP-1 could be functionally involved in the restriction of endometrial receptivity.

Intrauterine hCG infusion seems to be associated with endometrial synchrony and reprogramming of stromal development following ovarian stimulation. A study by Mansour et al., demonstrated that the administration of 500 IU of hCG to the endometrial cavity prior to embryo transfer on day 3 post-oocyte retrieval lead to statistically significant higher pregnancy and implantation rates compared to the control group. Pre-treatment with Hcg seems to have a beneficial effect on endometrial quality, defined as endometrial thickness >8 mm and assessed by ultrasound scan on the day of egg collection.

Besides increasing production of hCG by the trophoblastic tissue soon after implantation, this hormone is also produced by the blastocyst and may contribute in a paracrine manner to the

implantation process. Studies have shown that hCG represents the first known human-embryo derived signal in maternal-fetal communication, through which the embryo influences the immunologic tolerance and angiogenesis at the maternal-fetal interface.

Hcg and placentation

Recent research has focused on the connection of hCG to the evolution of human hemochorial placentation. Several studies have shown that the various forms of hCG were discovered with the appearance of hemochorial placentation. This communication system between the mother and the fetus is essential for the development of larger brains in humans and advanced primates. The presence of LH/CG receptors on spiral arteries in the myometrium and decidua and the role of hCG in the angiogenesis of these spiral arteries has been known for more than a decade. Using Doppler to study spiral arteries following in vivo hCG injections, Toth et al., demonstrated that hCG decreases vascular resistance in the vessels, causing dilation and promoting blood flow. A significantly larger variant of regular hCG, hyperglycosylated hCG is believed to play an important role in the establishment of hemochorial placentation. Hyperglycosylated hCG is produced by the invasive extravillus cytotrophoblast cells of the placenta in humans. It seems that its role lies in promoting invasion during placentation, while, on the other hand, it acts as an autocrine factor blocking apoptosis of these cytotrophoblastic cells. Based on these facts, Cole et al., suggested that hyperglycosylated hCG is responsible for the invasion of cytotrophoblast cells to the uterus and achieving an implantation that is as deep as possible, while regular hCG enhances spiral artery growth and multiplicity to meet and provide nutrition to the invading villi.

It is established knowledge that invasion distinguishes hemochorial placentation from more primitive placental models. However, obstetric complications are unique to humans, compared to other primates. Humans have developed a more sophisticated and complex model of placentation, in order to support the more demanding human brain. Moreover, it is estimated that nearly 41% of pregnancies in humans lead to miscarriages, early pregnancy losses and biochemical pregnancies, while this percentage declines to lower than 10% in most mammalian species. Sasaki et al., suggested that most pregnancy failures are due to inadequate hyperglycosylated hCG production on the day of implantation of the blastocyst. Researchers have also demonstrated that pregnancy-induced hypertension, preeclampsia and eclampsia are complications of incomplete hemochorial placentation mechanisms, which usually take place at the end of the first trimester of pregnancy. The possible association

between hCG and hyperglycosylated hCG with hemochorial placentation could lead to new cures and treatments for choriocarcinoma and invasive mole.

Hcg As An Anti-Rejection Agent

Based on the existence of a unique relationship between the mother and the fetus and given the important role of hCG in the establishment of this relationship, a new theory has been developed, which indicates that hCG could be the answer in the chronic rejection in solid organ transplantation. hCG promotes tolerance through a number of actions on the human immune system, while recently, it was found that hCG prolongs skin allografts in mice. Moreover, women receiving hCG preconditioning prior to IVF had reduced inflammatory IL17 but increased anti-inflammatory IL27 and IL10. In addition to this effect, the improvement in the symptoms of rheumatoid arthritis during pregnancy is due in part to the hCG induced shift of Th1 mediated cellular immunity to a pro-pregnancy Th2 immunity and an increase in T regulatory cell function. Therefore, it seems that these changes are in favor of both pregnancy and reduction in pathogenic RA immune activity. hCG has also been used with success in the management of paraneoplastic neuropathy mediated by anti-Hu antibodies.

Given the fact that the female organism enters a better state when pregnant, and women with minimally aggressive trophoblastic neoplasia and hCG levels over 3000 mIU/ml remain generally in good health it is a safe hypothesis that the use of hCG comes without any side effects and it is both subtle and specific in its action. This is an important difference between hCG and the use of the current anti-rejection agents, such as corticosteroids, ciclosporin, tacrolimus azathioprine, mycophenolate mofetil and a range of T-cell specific antibodies. Moreover, hCG could be used as a therapeutic agent against autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome. Being cheap and easy to use with minimum adverse effects, further studies need to be made in order to assess the efficacy of hCG on the treatment of autoimmune disorders.

Determination of biological activity of gonadotropin hormones

Determination of biological activity of gonadotropin hormones is essential in pharmaceutical manufacturing of the hormonal preparations which constitutes a unique approach to determine the functional aspects of gonadotropins. The aim of the present study was to asses the quantification of biological activity of Human chorionic gonadotropin (hCG) hormone which is commercially manufacturing at Sanzyme (P) Ltd and widely used in controlled

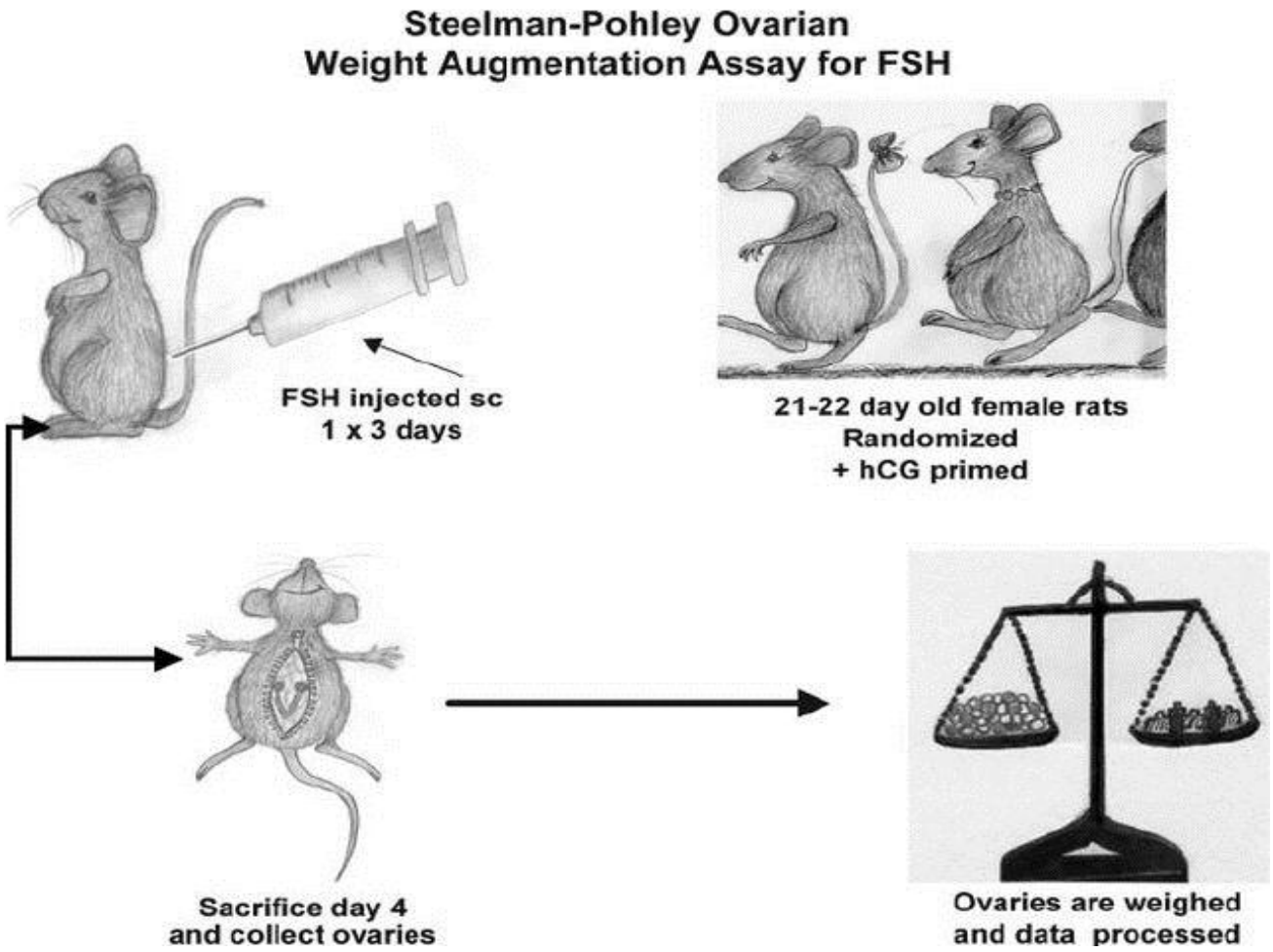
ovarian stimulation and induction of ovulation as key components of infertility treatment. A number of gonadotropin preparations are available, based on the naturally occurring gonadotropins: follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG).

Gonadotropins are glycoprotein hormones that regulate normal growth, sexual development, and reproductive function. These are large, up to 40 kDa proteins, which are synthesized and secreted by the gonadotropic cells of the anterior pituitary gland (LH and FSH) and by the syncytiotrophoblasts in the placenta (hCG). These hormones may vary in the level of glycosylation depending on myriad of physiological co-factors. Upon binding to FSH receptor (FSHR), a G-protein coupled receptor (GPCR), FSH regulates the development, growth, pubertal maturation, and reproductive processes, like maturation of germ cells in both women and men. LH and hCG bind to a shared GPCR (LH/CG receptor, LHCGR) and regulate mechanisms involved in ovulation, early pregnancy and placental function, respectively, in females, while in men, LH is involved in spermatogenesis and testosterone production.[1]

FSH, LH and hCG are heterodimeric proteins that consist of non-covalently linked α- and β-subunits. The gonadotropins share the α-subunit structure, but are each bound to a unique β-subunit resulting in differences in their physiological roles and signalling1. Upon binding to their receptor, gonadotropins activate the Gs-coupled signalling pathway resulting in stimulation of a family of cellular enzymes, adenylate cyclases. These enzymes catalyse production of the second messenger molecule cyclic adenosine monophosphate (cAMP) from cellular pool of ATP. cAMP in turn acts via its many effector proteins to result in a cellular response to receptor activation.

Over the last 50 years many assays have been developed for gonadotropin detection relying on *in vivo* physiological changes caused by gonadotropins on cells or animals, e.g. measurements of estradiol production in rat Sertoli cell culture after addition of FSH, testosterone production by mouse Leydig cells after LH or hCG stimulation or the linear relationship between administered gonadotropins and ovarian weight measured in rats post-mortem. In addition, many sensitive and very specific immunological assays have been introduced based on interactions of hormones with their specific antibodies.

In the current study we have used a conventional method for measuring the bioactivity of HCG which is first described by **Steelman-Pohley** which is also the mainstay of pharmacopiel monographs for the statutory determination of the potency of the urinary HCG preparations.



MATERIALS AND METHODS

Animal Husbandry

Housing

Animal room was cleaned before receiving of animals. During the study, the floor of the experimental room and work tops were swept and mopped with disinfectant solution every day. Cages were changed along with bedding material for weekly twice. Polypropylene cages with metal top grills were used for housing the animals. Three animals/sex/cage were housed during the entire study period.

Identification

Individual cage was identified with cage card. Acclimatization cage card was having details of study number, Test Item, species, strain, sex, animal number, acclimatization start and end dates. During treatment period, cage card was containing details of study number, test item, species, strain, sex, dose, animal number, date of treatment, date of necropsy. Individual animal was identified with tail marking method during entire study period.

Bedding

Autoclaved corn cob was used as bedding material. Details and analysis report of bedding material used was incorporated in the raw data.

Feed & water

Conventional laboratory rodent diet supplied by approved vendor was offered *ad libitum,* except, before dose administration animals were fasted overnight. Feed was provided for fasted animals after 3 hours of dose administration. Purified water was available *ad libitum* via drinking water bottles. Feed and water analysis reports were incorporated in raw data.

Environment Conditions

Animal holding room was maintained at controlled temperature and relative humidity of 20 to 25 °C and 50 to 60 % respectively throughout the study period.Throughout the study period 12 air changes were maintained in animals holding room. Artificial light was set to give a cycle of 12 hours light and 12 hours dark.

Acclimatization

Animals were acclimatized for 7 days. Animal body weight were recorded at the time of receipt and on last day of acclimatization. During acclimatization period animals were subjected for mortality check twice a day and cage side observations once a day. Detailed clinical observations were performed on day 7 of acclimatization.

Experimental design

Group No.	Group	Dose[#]	Conc.(IU/mL)	No. of Animals	Animal Strain	Animal b.wt/ Age
Y1	Workings Standard High dose	15 IU	0.2mL/ Total dose per rat 12 IU	8 Animals	Wistar Rats	25 - 35 Grams/21 Days old animals

Y2	Workings Standard Low dose	7.5 IU	0.2mL/ Total dose per rat 12 IU	8 Animals	Wistar Rats	25 - 35 Grams/21 Days old animals
Y3	Sample High dose	15 IU	0.2mL/ Total dose per rat 12 IU	8 Animals	Wistar Rats	25 - 35 Grams/21 Days old animals
Y4	Sample Low dose	7.5 IU	0.2mL/ Total dose per rat 12 IU	8 Animals	Wistar Rats	25 - 35 Grams/21 Days old animals

- Conc.: Concentration, No.: Number, mL: milli liter,b.wt.: body weight

CONCLUSION

In conclusion, the implemented assay for determination of biological activities of hCG and FSH, relying on hormone receptor activation , offers a valuable alternative for evaluation of different hormone preparations. We have demonstrated high sensitivity and reproducibility of the assay for hCG and FSH testing, which could potentially be used in clinical research and pharmaceutical industry.

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