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التوثيق الإلكتروني والميكروفيلم

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HANAA ALY

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Production and Evaluation of Local Radioimmunoassay System for Quantitative Estimation of Human Chorionic Gonadotropin in Human Serum

A Thesis Submitted for the Degree of Ph.D. in Biochemistry

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ABSTRACT

This study was focused on the overall production of in-house radioimmunoassay (RIA) system for quantitative estimation of human chorionic gonadotropin (HCG) in human serum. This work was accomplished by extraction and purification of HCG from the urine of pregnant women at the 10^{th} week of gestation as a basic material. Highly pure HCG was obtained with purity 98.2 %, recovery 60 %, and MW 36.7 kDa. All components of RIA were prepared, polyclonal (anti-βHCG) along with goat anti rabbit IgG 2^{ry} antibody, radio-iodinated HCG tracer, and HCG standards. Polyclonal anti-βHCG antisera were prepared by immunizing rabbits with βHCG. The obtained anti-βHCG antisera titer was $1/10^4$ with displacement 84.8 % and the goat 2^{ry} antibody titer was 1/50 with displacement 82.8 %. HCG tracer was prepared using optimized chloramine-T method, gave a yield 71.6 % and specific activity 170.42 µCi/µg. The purity of the tracer was 99.2 %. HCG standards were prepared in assay buffer matrix. All parameters were optimized, formulated and the standard curve was plotted. Validity was ensured, sensitivity 0.5 mIU/ml, cross-reactivity ~1%, intrassay and interassay precision with coefficient of variation (4.1 to 6.9 %) and accuracy test (recovery and dilution) (93.3 to 108.3 %). The results obtained provide an economic, highly sensitive and accurate HCG-RIA system can be used for pregnancy monitoring and diagnosis of gestational trophoblastic diseases.

INTRODUCTION

Human Chorionic Gonadotropin (HCG) is a member of the glycoprotein hormone family, which also includes human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH), and human thyroid-stimulating hormone (hTSH) **(Rodríguez *et al.*, 2005; Stenman *et al.*, 2006).** These heterodimeric hormones share a common α-subunit, but differ in their β-subunit, which confers biologic specificity **(Rodríguez *et al.*, 2005)**. The α-subunit of 92 amino acids is noncovalently associated with the hormone β-subunit. The β-subunit of HCG (βHCG) contains 145 amino acids **(Aldaz-Carroll *et al.*, 2015)**. The theoretical molecular weight of HCG is approximately 36.7 kDa **(Cole & Butler, 2010)**, the α subunit has a molecular weight of 14.5 kDa and the β subunit has a molecular weight of 22.2 kDa **(Fournier *et al.*, 2015)**. HCG is produced by the trophoblastic cells of the placenta and secreted as early as 7 days after fertilization with a cutoff value equal to 5 mIU/ml in serum, it plays a major role in maintaining the developing corpus luteum for progesterone production throughout the pregnancy **(L. Cole, 2009).**

During pregnancy, the concentrations of HCG become detectable 5–7 days after conception and increase exponentially, with a doubling time of about 1.5 days. Peak values are reached at 7–10 weeks of pregnancy. Then, the levels decrease slowly until the 15th week, after which there is a small gradual increase towards term **(Cole, 2010; Rodríguez *et al.*, 2005).**

The primary function of HCG is the regulation of pregnancy development and its maintenance. Under physiological conditions, HCG is responsible for various processes such as implantation,

placentation, angiogenesis, embryo development, and immune tolerance **(Cole, 2013)**. Thus, the measurement of HCG concentration is a powerful tool for pregnancy monitoring **(Butler *et al.*, 2013).** The difference in hormone level marks some pathological conditions like failing pregnancy, ectopic pregnancy, hydatidiform mole, gestational trophoblastic diseases, choriocarcinoma, and germ cell malignancy **(Głodek *et al.*, 2012; Iles *et al.*, 2010)**. However, in urine and serum of both pregnant women and tumor patients, various forms of HCG were identified **(Cole, 2012)**. These forms may be used as disease-specific markers. Unfortunately, commercially available assays fail to detect and distinguish among these forms. Thus, a detailed analysis together with new test development is needed **(Szczerba *et al.*, 2016)**.

Radioimmunoassay (RIA) is the most recognized sensitive microanalytical technique for the assessment of a wide range of substances of biological and medical interest. RIA employs principles of immunology thus making the reaction very specific, and uses radioactive substances which can be counted with great sensitivity and accuracy **(Abu-Bakr El-Bayoumy *et al.*, 2018)**.

The inherent problem with most market immunometric assays is that they must have antibodies to at least two distant sites on the HCG molecule, the immobilization antibody or antibodies, and one of the tracer antibody or antibodies. Fortunately, the RIA needs just one antibody to core β-subunit-1 or β-subunit-2 and can measure all variants of HCG. The immunometric assay with its requirement of distant antibodies can only measure variants that have the two antibody epitopes. This very much limits what can be detected and measured **(Cole, 2015b).**

The principle of HCG-RIA is based on the competitive binding of serum HCG and a fixed amount of radioactively labeled HCG (^{125}I-HCG) for a limited number of determinants on specific βHCG-Ab (polyclonal). The formed immune complexes are precipitated with a combined immunoadsorbent formula (secondary antibodies & polyethylene glycol) and separated out. The amount of radioactivity, measured in a gamma scintillation counter, is inversely proportional to the concentration of HCG in the examined specimen **(Cole, 2015c).**

The adoption of "in house" assays that use locally prepared reagents-based methods in contrast to "ready to use" commercial kits has proved to be the key to success in RIA projects, especially in developing countries, where the cost is often a decisive consideration. Replacing commercial kits with locally prepared basic reagents, even when the raw materials are all imported from a central source, has reduced the cost of analysis, Local production of primary reagents also contributes towards the achievement of self-sufficiency in reagent supplies.

Aim of the work:

The overall objective of this study is to assess the technical as well as the economic feasibility of establishing sensitive, specific and accurate “in-house” radioimmunoassay (RIA) system for human chorionic gonadotropin (HCG) based on locally prepared reagents. This will lead to significant cost reduction and extended use for routine pregnancy monitoring and diagnosis of gestational trophoblastic diseases.

I. REVIEW OF LITERATURE

I.1. Human Chorionic Gonadotropin

I.1.1. Historical background

In 1912, Bernhard Aschner, a gynecologist at the University of Vienna, stimulated the genital tract of guinea pigs by injecting water-soluble extracts from the human placenta **(Aschner, 1912)**. In 1913, Otfried Fellner, a gynecologist in Vienna, induced ovulation in immature rabbits by injecting saline extracts from the human placenta **(Fellner, 1913)**. In 1919, Toyoichi Hirose, a scientist at the Osaka Medical College, stimulated ovulation in immature rabbits by repeated injection of extracts from the human placenta **(Hirose, 1919)**. All of these works show that there is a clear hormonal signal between the placenta and the uterus. In 1927, Selmar Aschheim, a gynecologist at the University of Berlin, and Bernhard Zondek, a gynecologist at the Berlin-Spandau Hospital demonstrated that pregnant women produce a gonad-stimulating substance. They showed that injecting this substance subcutaneously into immature female mice produced follicular maturation, luteinization, and hemorrhaging into the ovarian stroma **(Aschheim & Zondek, 1927)**. These findings were later confirmed by Friedman and Lapham in 1931 and hence the first HCG/ pregnancy test was born **(Friedman & Lapham, 1931).**

These early tests primarily used urine to promote ovulation in mice and rabbits **(Delfs, 1941; Friedman & Lapham, 1931; Galli-Mainini, 1948; Kupperman *et al.*, 1943)** and were commonly referred to as the “rabbit” or Friedman test. During the next four decades, bioassays like the rabbit test were the only

practical way to detect pregnancy or measure HCG. In 1960, the first antibody-based tests hemagglutination inhibition and latex agglutination were used **(Wide, 1962; Wide & Gemzell, 1960)**. These tests were insensitive slide tests that detected HCG at a concentration of 1000 mIU/ml or greater.

In 1964, the competitive radioimmunoassay (RIA) for HCG was invented and revolutionized pregnancy testing. A sensitive test was available that could measure HCG as low as 5 mIU/ml, and it could measure pregnancy close to the day of a missed period. The invention of the RIA led to readily available pregnancy testing and HCG quantification at clinical laboratories throughout the world **(Aono *et al.*, 1967; Lunenfeld & Eshkol, 1967; Paul & Odell, 1964; Rushworth *et al.*, 1968; Vaitukaitis *et al.*, 1972; Wilde *et al.*, 1965)**. The initial Radioimmunoassay was problematic because it used an antibody against HCG dimer and detects both HCG and LH. The identity between the α-subunit of HCG and that of LH and the 80 % homology in the β-subunit of HCG and LH was the cause of invalidity of the initial HCG dimer RIA. So that, the early HCG dimer RIA can detect both HCG and LH and, thus, could only demonstrate pregnancy and exclude LH by showing a continual increase in hormone levels.

In 1972, Vaitukaitis et al. introduced the βHCG-RIA test **(Vaitukaitis *et al.*, 1972)**, a pregnancy test using an antibody against the β-subunit of HCG. The βHCG test was the first HCG-specific RIA. Unlike the initial HCG dimer test, which detected both HCG and LH, the βHCG test measured HCG alone and did not detect LH **(Vaitukaitis *et al.*, 1972)**. This modification was an important elimination of LH, which was not involved in the

pregnancy and hence no need to be measured when testing for pregnancy. The βHCG-RIA test became the world standard for the next 20 years. Even today, in the age of immunometric assays, both physicians and textbooks still describe HCG tests as βHCG tests.

The discovery of monoclonal antibodies in 1975 was paramount to the development of the modern immunometric HCG tests **(KÖHler & Milstein, 1975)**. The advanced two- or three-antibody immunometric HCG assays were developed in 1981, dual-antibody immunometric technologies are the principle of most modern point-of-care rapid pregnancy tests in the physician's office and in-home or over the counter rapid pregnancy tests **(Joshi *et al.*, 1981)**. A positive result in these tests is indicated by a line formed in the plastic window by the immobilized antibody HCG dye antibody complex.

I.1.2. Structure, synthesis and secretion of HCG

- **Structure of HCG**

HCG is a complex glycoprotein with a molecular weight of 36.7 kilo Dalton (KDa) **(Gam & Latiff, 2005)**. It is composed of two glycosylated subunits α and β which are non-covalently associated. The α-subunit is identical to the pituitary hormones; (LH, FSH, and TSH). It contains 92 amino acids with two N-glycosylation sites, and is encoded by a single gene (CGA) located on chromosome 6q21.1-23 **(Fournier *et al.*, 2015)**. The β-subunits are distinct in each of the hormones and confer receptor and biological specificity. The HCG β-subunit contains 145 amino acids with two sites of N-glycosylation and four sites of O-glycosylation, and is encoded by six non-allelic genes (CGB) clustered on

chromosome 19q13.3 and named CGB1, CGB2, CGB3, CGB5, CGB7, and CGB8 **(Rull *et al.*, 2007)**.

HCG is a highly glycosylated molecule. 70% of its structure is represented by the peptide and 30% by carbohydrate residue. The sugar branches are covalently bound to the peptide chains, it consists of an O-linked oligosaccharide containing an N-acetylgalactosamine residue linked to a serine residue and an N-linked oligosaccharide containing an N-acetylglucosamine residue linked to an asparagine residue. The alpha subunit contains 2 N while the beta subunit contains 2 N and 4 O glycosylation sites. The secretion, biological activity, and half-life of HCG are highly dependent on the glycosylated state of the molecule (micro heterogeneity due to the variability of oligosaccharide moiety). The sialic acid content of HCG has a major significance in its receptor binding ability, biological activity, and clearance from the maternal circulation **(Fournier, 2016)**.

- **Synthesis and secretion of HCG forms**

The HCG amino acid sequence supports 5 glycoproteins, all are called HCG forms. The first of the five HCG molecules is a dimeric glycoprotein of molecular weight 36.7 KDa made by placental syncytiotrophoblast cell. It is an unusually acidic glycoprotein with an isoelectric point (pI) of 3.5. Acids are negatively-charged molecules at physiological pH that are repelled by the glomerular basement membrane, which is also negatively charged. The acidity also gives HCG a long-circulating half-life of 36 h. HCG has multiple hormonal functions during pregnancy, ranging from maintenance of corpus luteal progesterone production to promotion of growth and differentiation of the uterus, placenta,

and fetus, growth of uterine and umbilical blood vessels and blockage of macrophage destruction by maternal tissues of fetoplacental components as foreign tissues **(Cole & Butler, 2010)**.

On the other hand, sulfated HCG, the second HCG-related molecule is produced by the pituitary gonadotrope cells. Sulfated HCG production seemingly follows LH production in both men and women, as it functions to supplement LH actions. Interestingly, sulfated HCG is produced at approximately 1/50th the level of LH **(Cole & Gutierrez, 2009; Odell & Griffin, 1987, 1989)**. Yet HCG has 50-fold greater biopotency (circulating half-life) than LH **(Birken *et al.*, 1996).** It is concluded that sulfated HCG may be the co-promoter of ovulation, androstenedione production in the follicular phase of the menstrual cycle, and progesterone production in the luteal phase of the menstrual cycle.

Hyperglycosylated HCG is the third HCG-related molecule. It is produced by the root cytotrophoblast cells and is autocrine that promotes implantation during pregnancy **(Cole, 2010; Sasaki *et al.*, 2008)**. Hyperglycosylated HCG promotes invasion of the uterus at pregnancy implantation, it also promotes the growth of placental tissue during pregnancy. Hyperglycosylated HCG also drives the growth and malignancy of choriocarcinoma and germ cell malignancies. These are cancers of cytotrophoblast tissue, or cancers that take on cytotrophoblast histology **(Cole, 2010; Cole *et al.*, 2006)**. Previous studies indicate that hyperglycosylated HCG antagonizes a TGFß receptor on cytotrophoblast cells **(Butler *et al.*, 2000; Cole & Butler, 2012)**.

Indeed, all advanced cancers (except choriocarcinoma and germ cell malignancies) produce HCGß and/or hyperglycosylated HCGß in variable proportions **(Acevedo & Hartsock, 1996),** the fourth and fifth HCG molecules respectively. These molecules are known to be major cancer promoters, promoting advanced cancer growth and malignancy by antagonizing a TGFß receptor, like the hyperglycosylated HCG **(Butler *et al.*, 2000; Cole & Butler, 2012)**.

I.1.3. Physiological functions of HCG in pregnancy

- **Preimplantation and blastocyst formation**

HCG is the first hormonal signal of the conceptus. Its mRNA is transcribed as early as the 8-cell stage **(Jurisicova *et al.*, 1999)**, while the blastocyst expresses HCG before its implantation. HCG is exponentially produced after implantation by the syncytiotrophoblast. Significant levels of HCG can be measured in the maternal blood 10 days after fertilization. The peak of HCG production is reached between the 10^{th} and 11^{th} week of gestation; the production then decreases to remain steady. HCG mediates rescue of the corpus luteum and ensures the ongoing production of progesterone **(Makrigiannakis *et al.*, 2017; Shikone *et al.*, 1996)**.

Blastocyst cells and placental cells may be singular as cytotrophoblast cells, or fused cells; 3 to 50 cells fused together as syncytiotrophoblast cells **(Figure I.1.)**. These syncytiotrophoblast cells make the HCG molecule and place short sugar side chains on its structure. This is the HCG hormone. Meanwhile, the unfused cytotrophoblast cells make the amino acid structure HCG and put large sugar side chains on it (39% of molecular weight). This is the