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Negative Pregnancy Test in Patients with Trophoblastic Diseases

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Abstract Quantitative and qualitative human chorionic gonadotrophin (hCG) assays are widely used to detect pregnancy state and abnormal trophoblastic lesions. At least five different forms of hCG have been characterized and different trophoblastic diseases produce different forms of hCG in varying proportions. Because of the difference in antibody specificity in various commercial automated immunoassays of HCG, discordant results may be obtained by laboratories using different hCG assays, with a falsely low or negative result obtained if the assay does not recognize the hCG variants produced from the trophoblastic tissue. On the other hand, significantly elevated hCG concentration can paradoxically lead to false-negative results in two-site immunometric assay due to high-dose hook effect. Clinicians managing patients with trophoblastic lesions should be aware of these limitations of current hCG assays and clinical laboratories should have measures to avoid analytical false negative hCG results.

Keywords Human chorionic gonadotropin · Gestational trophoblastic disease · High-dose hook effect · Immunoassay · Pregnancy test · Point-of-care testing · Trophoblastic diseases · Negative pregnancy test

Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone consisted of two noncovalently linked α and β subunits. The β subunit is unique to hCG and confers specificity in immunoassay. Currently, five different forms of hCG have been characterized, including regular hCG produced by syncytiotrophoblast, hyperglycosylated hCG (hCG-H) produced by cytotrophoblast, hCG free β-subunit (hCGβ) together with hyperglycosylated hCGβ produced by various malignancies, and sulfated hCG produced by pituitary [1]. Each has distinct molecular structure and biological function.

Our knowledge on biological functions of hCG in various conditions has expanded dramatically, from textbook teaching of hCG promoting progesterone production from corpus luteal to hyperglycosylated hCG's participation in pathogenesis of choriocarcinoma cells through stimulating growth and invasion [2]. Recent research has suggested that hCGβ blocks apoptosis and promotes growth in trophoblastic and nontrophoblastic malignancies [3, 4]. Not surprisingly, hCG assays have extended its role beyond a "pregnancy test," meaning detection of normal pregnancy, estimation of gestational age, detection of miscarriages, or ectopic pregnancy. It is now used as a tumor marker for gestational trophoblastic diseases (GTD) [5], seminomas, teratomas, and a number of other nontrophoblastic ectopic hCG-secreting cancers, such as bronchogenic carcinoma. Indeed, a total hCG level >100,000 mIU/mL is regarded as strongly suggestive of GTD, particularly complete hydatidiform mole. Although it should be noted that the peak serum hCG level also may reach such level at approximately 8 to 10 weeks gestation in normal pregnancy. Patients with partial moles usually present with a lower level of serum total hCG. The USA hCG Reference Service's data on the median levels of serum total hCG in normal pregnancy and gestational trophoblastic diseases is summarized in Table 1 [6].

Because of such diverse clinical uses, a wide variety of qualitative and quantitative hCG assays produced by different assay manufacturers are available in the market. Virtually all commercial hCG assays are immunoassay based, which

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Table 1 Comparison of serum total hCG in normal pregnancy and gestational trophoblastic diseases [6]

Source	n	Total hCG mIU/mL median
4 weeks gestation	16	239
9 weeks gestation	7	128,300
27-40 weeks gestation	49	21,025
Complete mole (before evacuation)	30	192,995
Partial mole (before evacuation)	21	48,900
Highly invasive choriocarcinoma (>50 % hCG-H)	17	45,350
Invasive choriocarcinoma (<50 % hCG-H)	44	4,258
Placental site trophoblastic tumor (at time of diagnosis)	21	30

basically means detection of the presence or measurement of concentration of an analyte in a biological fluid through the use of antibodies. Qualitative hCG assays are mainly used as over-the-counter or point-of-care testing devices to rapidly establish or rule out a diagnosis of pregnancy, whereas quantitative hCG assays are mainly used by clinical pathology laboratories utilizing automated immunoassay analyzers. When clinical laboratories or clinicians choose an assay to measure hCG, they need to know the indication of measuring hCG and forms of hCG measured by these assays because not all hCG assays are suitable for use in management of trophoblastic diseases. Also, the design and analytical principles of the hCG assay should be studied because certain types of immunoassays are intrinsically susceptible to high-dose hook effect. In this article, causes of false negative hCG test due to such analytical issues would be discussed.

High-Dose Hook Effect Causing False-Negative hCG Test

An important cause of falsely low hCG assay is high-dose hook effect, also called prozone effect. It is a well-known phenomenon in the field of clinical chemistry, especially in assays when analytes present in samples in extremely wide range of concentrations, such as tumor markers [7, 8], hormones [9, 10], and immunoglobulins [11]. It was first described almost four decades ago in a two-site immunoradiometric assay by Miles et al. [12] where a paradoxical fall in dose–response was observed at high ferritin concentration.

To understand high-dose hook effect, the analytical methodology of hCG assays will be discussed first. Both qualitative and quantitative hCG assays used nowadays are two-site noncompetitive immunometric assays, also known as "sandwich" assay. In this assay design, a "capture" antibody, which is bound to a solid phase first captures the hCG in sample. Then, a "signal" antibody, which contains a label for detection, such as enzymes, colour tag, fluorescent, chemiluminescent labels, etc., would recognize and bind to a separate epitope on hCG molecule. As a result, hCG molecules link up both capture and signal antibody to form the sandwich (capture antibody-hCG-signal antibody). Because the concentration of hCG molecules is directly proportional to the bound signal in the assay system, the hCG level in the sample can be obtained by comparing the signal of label in the sample to that of calibrators with known concentration of hCG standard. However, when hCG in the sample is present in huge excess, it will simultaneously bind to and saturates all the available binding sites on both capture and signal antibodies, preventing the formation of sandwich, causing a falsely low level of hCG measured or even down to undetectable level [13].

Recognizing this limitation of two-site immunometric assay, assay manufacturers have introduced ways to avoid hook effect. Increasing the quantity of capture and signal antibody is one way to extend the linearity range of assay to a higher analyte level. However, this would still be susceptible to hook effect at extremely high level of analyte. An alternative way to alter the sample antigen to reagent antibody ratio is to perform serial dilution of the sample. Indeed, a paradoxically higher result obtained in diluted sample compared with undiluted sample is a simple way to detect hook effect. Various dilution protocols have been published [14, 15], and clinical laboratories should have standard protocol for performing such procedure in samples of suspected falsely low results. Finally, a wash step can be introduced between reaction of analyte with solid-phase capture antibody and addition of signal antibody. This wash step will remove the excess analyte and avoid signal antibody from being saturated by excess analyte. However, this would add an extra step in each sample analysis, which compromises speed and throughput of the analyzer. So although two-step assay design is analytically sound and preferable, it has not been adopted in many immunoassay analyzers.

Al-Mahdili et al. have studied the occurrence of high-dose hook effect in six commercial hCG immunoassays [16]. They demonstrated that four of six commercial assays are susceptible to hook effect and they are one-step assays, whereas the remaining two assays, which did not show any hook effect, are two-step assays. Also, the four one-step assays produced the falsely low results without any warning flag. This clearly suggests that the error may not be identified unless the laboratory is alerted of the incompatible clinical context and performs additional investigation, such as a re-run of hCG assay after sample dilution. Indeed, cases of falsely low hCG results due to high-dose hook effect have been repeatedly reported in patients with gestational trophoblastic diseases, which can lead to mismanagement due to delay in diagnosis or misdiagnosis [17–21]. In one case report, unnecessary hysterectomy was performed for a patient presenting with intermittent vaginal bleeding, because the diagnosis of choriocarcinoma was not made based on a falsely low serum hCG

result [17]. Furthermore, it is cannot be overemphasized that point-of-care and over-the-counter qualitative "pregnancy test" hCG devices also are, if not more, susceptible to high-dose hook effect simply, because they are one-step sandwich immunometric assays [22]. Multiple case reports of false-negative point-of-care pregnancy test have been published in patients with choriocarcinoma [23, 24], molar pregnancy [25–29], hyperemesis gravidarum [30], and even normal pregnancy [31]. Most of the cases were reported by emergency physicians, and this probably reflects the widespread use of point-of-care pregnancy test device in accident and emergency department. However, these point-of-care hCG devices may be used by clinical staff who are unfamiliar with the analytical principle and the possibility of hook effect. Failing to recognize this limitation of assays can lead to serious consequence as shown by these case reports.

Recently, a variation of hook effect called the "variant hook effect" was reported by Gronowski et al. [32]. In this phenomenon, an excess of core fragment of hCGβ (hCGβcf), which is a degradation product of hCG and is the predominant form of hCG in urine of women in later part of pregnancy, was found to cause false-negative result in some commercial point-of-care qualitative hCG devices. They suggested that because the point-of-care pregnancy test device used in their center was designed for detection of early pregnancy, the device only recognizes intact and nicked hCG but not other variants, including hCGβcf. In the presence of excess hCGβcf, binding sites of either one of the capture or signal antibody are saturated by hCGβcf, preventing formation of a "sandwich" and detection of other forms of hCG, such as intact hCG. The authors also noted that numerous cases of faint positive results were obtained for urine specimens from women at 5 to 8 weeks gestation, which would turn positive after dilution of specimen. This “variant hook effect” also highlights the importance of analytical specificity of immunoassays on hCG test results, which is discussed in the next session.

Effect of Analytical Specificity of hCG Assays on Diagnostic Sensitivity

It has long been recognized that different commercial quantitative hCG assays results are not directly comparable; this has been demonstrated by the wide interlaboratory difference in hCG measured result on the same sample in external quality assurance program. The underlying reason for this variation is that even if the assays are traceable to the same WHO hCG International Standard, different commercial hCG assays utilize different polyclonal or monoclonal anti-hCG antibodies. As discussed previously, five different isoforms of hCG are produced by different tissues in distinct clinical scenarios. To further complicate the matter, intact hCG molecules are metabolized by tissue of origin into free subunits and various nicked and cleaved forms [33]. Studies have shown that this degradation process is even more pronounced in GTD compared with normal pregnancy [34, 35]. As a result, hCG in blood and urine is a highly complex and heterogeneous mixture of different isoforms and their metabolites. Despite the fact that many manufacturers claims their hCG assays are "total hCG assays," most do not provide information on the forms of hCG that are recognized by their methods. Many studies have been conducted to determine the analytical specificity of currently used commercial hCG immunoassays, using WHO International Standards or International Reference Reagents [36, 37] or standards from other sources [38–40]. All these studies showed significant variation in the spectrum of forms of hCG that are recognized by various commercial hCG assays. Such variability explain the considerable difference in hCG results obtained from different laboratories for the same patient sample or external quality assurance program material, despite the fact that most assays currently are calibrated against the 4th WHO International Standard (IS 75/589). These studies have two clinical implications: First, serial monitoring of serum hCG levels in the same patient should be done using the same laboratory or laboratories with the same hCG assay platform. Otherwise, the serial serum hCG results are not comparable even though the same unit of IU/L is reported by different laboratories. Second, some hCG assays are not suitable for use as tumour marker for management of trophoblastic or nontrophoblastic neoplasms. The above-mentioned studies have clearly demonstrated that some commercial hCG assays grossly underdetect certain isoforms of hCG. For instance, an hCG assays that fail to recognize hCG-H would potentially miss the diagnosis of invasive mole and choriocarcinoma, in which the predominant form of hCG secreted by these lesions is hCG-H [41, 42]. Whereas recognition of hCGβ is essential for diagnosing placental site trophoblastic tumor [43] and nontrophoblastic hCG-secreting tumors, such as seminoma [44]. Indeed, cases of missing a diagnosis of persistent or recurrent trophoblastic neoplasm due to inability of hCG assays to fully detect certain hCG isoforms have been reported [45–48], and this can potentially lead to serious clinical and even medicolegal consequence. However, many gynecologists may not be aware that commercial hCG assays are FDA-approved for diagnosis of pregnancy only, and the current use of hCG as tumor marker is actually "off-label" and may put clinicians and clinical laboratories liable in case of litigation. Therefore, it is the responsibility of clinical laboratories to seek information on the spectrum of hCG isoforms that their immunoassays cover. Given the fact that most assay manufacturers do not provide such information in the kit insert, the above-quoted studies should be consulted in order to choose a "total" hCG assay that has board specificity and fit for use as tumor marker. Oncologists managing patients with trophoblastic neoplasms

also should be aware of the heterogeneity of hCG molecules and know which hCG assay their laboratories are using.

Conclusions

The almost ubiquitous adoption of commercial hCG assays in automated analyzers by clinical laboratories is largely driven by the cost, speed of analysis, and throughput. Also, the need to rapidly rule out pregnancy state has lead to introduction of point-of-care urinary pregnancy test kits in hospital emergency departments and outpatient clinics. Before using these hCG assays to manage patients with gestational trophoblastic diseases and other trophoblastic lesions, it is critical for both clinical chemists and clinicians to realize that commercial hCG assays, which are FDA-approved for diagnosis of pregnancy, are subject to false-negative results due to (1) high-dose hook effect and (2) failure to recognize certain isoforms of hCG. Studies on the susceptibility to high-dose hook effect and analytical specificity to hCG isoforms in various commercial hCG assays have been published and should be a good starting point to decide whether a certain assays is fit for purpose. When facing a negative hCG test result that is incompatible with other clinical features, such as characteristic ultrasound appearances of hydatidiform mole, laboratories should be informed of such discrepancy and initiate further investigations, including rerun hCG assay after dilution or sending the specimen to laboratories utilizing total hCG assays with board specificity. Clinical acumen, knowledge on analytical limitations of hCG immunoassays, and close communication between clinicians and clinical chemists are essential to prevent misdiagnosis, mismanagement, patient complaints, and lawsuits.

Compliance with Ethics Guidelines

Conflict of Interest Chun-Wing Yeung and Annie NY Cheung declare that they have no conflict of interest.

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