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Background Proteins in Human Chorionic Gonadotropin Pharmaceutical Formulations of Different Origins

Tanja Panić-Janković and Goran Mitulović

Abstract

Gonadotropins, including human chorionic gonadotropin (hCG), have been used since and for several decades to treat infertility by ovarian stimulation. hCG is the most important protein for embryogenesis and embryo development and implantation in uterus upon fertilization of oocytes. The hCG used for in-vitro fertilization (IVF) is being extracted from urine of pregnant women, and it does inevitably contains other proteins secreted into urine. The presence of other proteins varies from batch to batch, and it can be significantly high. Due to the fact that many of the proteins identified in these formulations can trigger an allergic reaction, which, in turn, can affect the embryogenesis and prevent embryo implantation, it is very important to check the amount and type of contaminant proteins in pharmaceutical formulations. It was found that the total protein content varied from batch to batch, and a large number of contaminant urinary proteins were identified in all analyzed samples except for the recombinant product.

Keywords: embryogenesis, in-vitro fertilization, human chorionic gonadotropin, proteomics

1. Introduction

Human chorionic gonadotropin (hCG) is one of the most widely studied markers in embryonic development. It is used as an obstetric marker, and it is often regarded as little more than a signal for maternal recognition of pregnancy. Human chorionic gonadotropin is a member of the dimeric glycoprotein hormone family that also includes FSH, LH, and TSH. The members of this hormone family share a common α subunit and have a unique β subunit to each hormone. Additionally, each hormone shows a different level of glycosylation, which determines circulating half-life and receptor binding affinity [1].

The success of embryo implantation upon IVF and embryo transfer depends on various factors related to the embryo quality and patient's endometrial receptivity. Upon implantation, it is important that the embryo reaches the endometrial cavity during the period of time in which the endometrium is receptive. It has been estimated that 50–75% of lost pregnancies are due to the embryo's implantation failure as described by Tsampalas et al. [2]. Many factors are involved in the implantation

process, which is very intricate process and the success can be influenced by many factors. The most important regulation of embryogenesis and embryo implantation in uterus is performed by hCG, and studies performed by Licht et al. [3] showed that an intrauterine injection of 500 IU of hCG/mL inhibited the expression of intrauterine insulin-like growth factor-binding protein 1 and the macrophage colony stimulating factor. It was also demonstrated that an intrauterine injection of 500 IU of hCG performed before embryo transfer significantly improved both the implantation and pregnancy rates in IVF/intracytoplasmic sperm injection cycles.

The use of gonadotropin derived from either animal or human tissues was not always without clinical danger (e.g., antibody formation from pregnant mare serum gonadotropin and Creutzfeld-Jacob disease from human pituitary gonadotropin).

The hCG is being extracted from urine of pregnant women (uhCG) for almost three decades, and it is being used for induction of mid-cycle follicular maturation and ovulation in women undergoing an IVF treatment. Originally, hCG in pharmacological preparations was derived only from the urine of pregnant women. However, due to their biological origin, these hCG products show large biological variability and significant batch-to-batch variation. Therefore, recombinant technology has been introduced for the production of recombinant hCG (rhCG) with higher purity and higher batch-to-batch reproducibility and the possibility to control their availability in different doses. The availability of different amounts of active substance in recombinant products provides a good starting point to develop personalized therapy for patients depending on their individual hormonal status.

Although a recombinant product is available on the market, urinary preparations of this hormone are still manufactured and are widely used [4–9]. Often, the urinary preparations are associated with problems arising from the fact that the starting material might originate from unknown sources, have poor purity, and lead to large batch-to-batch variations in activity and the amount of other proteins.

Analysis of commercially available, uhCG, was performed earlier [8], and discussions about the possible risks of infection were published [9–15].

We have analyzed several batches of both urinary derived and recombinant hCG formulations and have compared the obtained results in terms of number of identified proteins and their function during the embryogenesis.

2. Materials and methods

2.1 Analyzed hCG: source of the material

Different batches of both uhCG and rhCG were purchased through the pharmacy of the General Hospital of Vienna and by direct purchase from pharmacies in Bosnia-Herzegovina and Serbia. Details on manufacturer and batches analyzed are shown in **Table 1**.

2.2 Proteomics sample preparation

Trypsin for protein digestion was purchased from Promega Inc. (Vienna, Austria). Solvents for HPLC—methanol (MeOH), acetonitrile (AcN), 2,2,2-trifluoroethanol (TFE), formic acid (FA), heptafluorobutyric acid (HFBA), iodoacetamide (IAA), triethyl bicarbonate (TEAB), and dithiothreitol were purchased from Sigma-Aldrich (Vienna, Austria). Digestion of hCG and FSH was performed using the routine approach described in earlier publications [16].

Commercial name and charge number	Dosage	Principal component	Origin	Manufacturer
Pregnyl_M038101	5000 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Pregnyl_M011526	5000 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Pregnyl_354043	5000 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Predalon_117730	5000 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Pregnyl_117447	5000 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Pregnyl_116935	1500 IU/mL	hCG	Urinary-derived	MSD (N.V. organon, NL)
Pregnyl_M018720	5000 IU/mL	hCG	Urinary-derived	MSD
Chorimon_160432	5000 IU/mL	hCG	Urinary-derived	Institut Biochimique SA, CH
Ovitrelle	250 µg/0.5 mL	hCG	Recombinant	Merck Serono (Feltham, Middlesex, UK)

Table 1.
Analyzed products, batch numbers, and manufacturers' names of analyzed samples.

2.3 Chromatographic separation and detection

All separations were performed using the nanoRSLC UltiMate 3000 HPLC system coupled to the Q-Exactive Orbitrap Plus mass spectrometer (ThermoScientific, Vienna, Austria). Digested hCG and FSH were separated using trap column for sample loading and focusing (Acclaim PepMap C18, 300 µm ID × 5 mm, ThermoScientific, Vienna, Austria) and the pillar-arrayed-column (µPAC) with 2 µm interpillar distance and 2 m separation path (PharmaFluidics, Gent, Belgium) as the separation column. Both columns were operated in the column oven at 50°C. The sample was loaded onto the trap column using aqueous 0.01% HFBA at 30 µL/min and separated using the gradient generated by mixing mobile phases A (95% water, 5%AcN, and 0.1%FA) and B (50%AcN, 30%MeOH, 10% TFE, 10% water, and 0.1%FA).

Detection was performed using both UV at 214 nm and MS using positive electrospray ionization with a nanosource and ionization needle of 20 µm ID and 10 µm tip. The 20 most intensive signals in each MS scan were selected for MS/MS (fragmentation) with HCD at normalized collision energy (NCE) set to 30.

2.4 Data analysis

Raw data were transformed into Mascot generic files (MGF) for database search using MSConvert (www.proteowizard.sourceforge.net). The database search was performed using the in-house Mascot server v.2.6 and the SwissProt database (status January 2018) using following parameters: trypsin was selected as enzyme, peptide mass precision was set to 10 ppm, carboxymethylation on Cys was selected as fixed modification and oxidation on Met, and phosphorylation on Ser, Thr, and Tyr were set as variable modifications.

Pathway analysis was performed using String (www.string-db.org).

3. Results

3.1 Identification of contaminant proteins in hCG preparations

All sample preparations were performed using standard procedures used in the Proteomics Core Facility of the Medical University of Vienna. No gel separations have been performed, and all results are generated upon the in-solution digest of different commercial formulations. Following chromatographic separation of tryptic peptides, the detection was performed using nanoelectrospray positive ionization and database search using the common SwissProt database.

For all analyzed samples, active substances were identified as major compounds. However, a number of other proteins were also identified in all samples. **Figure 1** shows an exemplary total ion chromatogram (TIC) of an hCG sample.

Table 2 shows the total number of identified compounds in each of the analyzed sample in addition to the main sample component, and an overview of the proteins with the highest scores identified in all samples is shown in **Table 3**.

3.2 Contaminant proteins in rhCG preparations versus uhCG

Recombinant hCG was significantly cleaner but a certain number of other proteins were also identified. Although the majority of these proteins seem to be

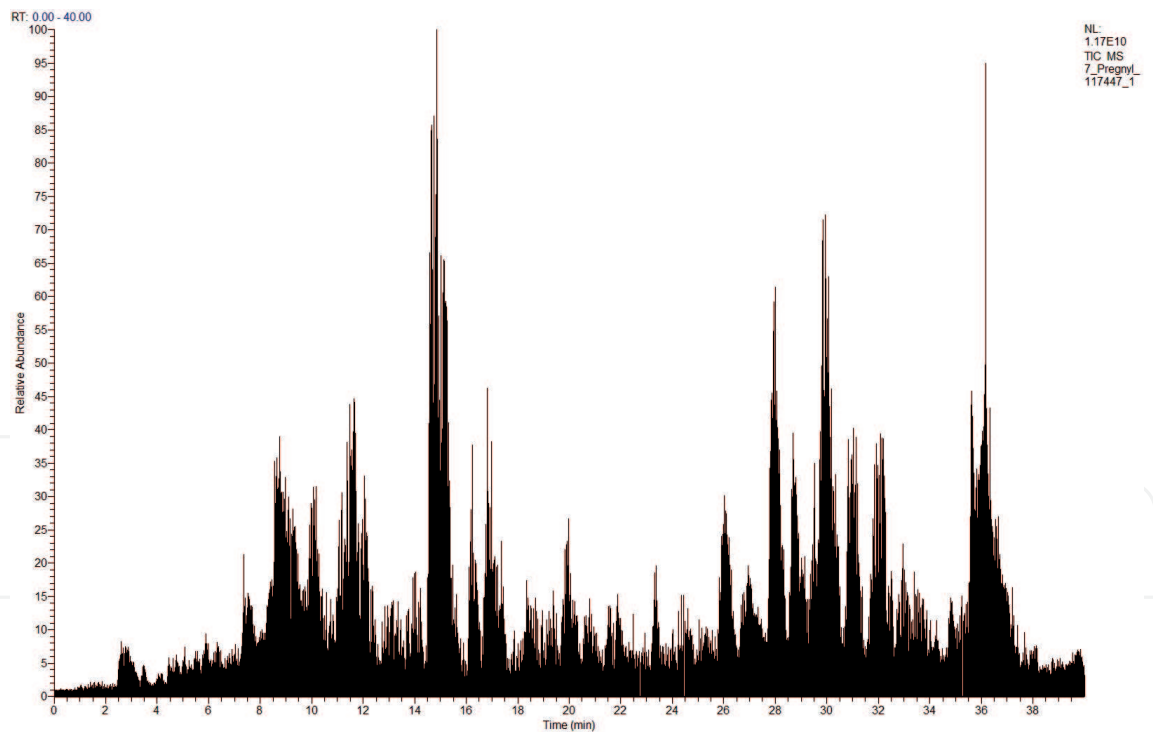


Figure 1.
Total ion chromatogram (TIC) of tryptic peptides generated from a Pregnyl formulation.

Sample's commercial name	Pregnyl	Chorimon	Ovitrelle
Number of analyzed batches	7	1	1
Origin	Urine	Urine	Recombinant
Number of additionally identified proteins	383	21	9

Table 2.
Total number of proteins identified in addition to the main therapeutic component in each of the samples' groups.

u-hCG	u-hCG-HP	r-hCG
Uromodulin	Growth/differentiation factor	Serum albumin
Ribonuclease pancreatic	Peptidoglycan recognition	Prelamin-A/C
Nonsecretory ribonuclease	Protein 1	Keratin, type I
Apolipoprotein D	Prostaglandin-H2 D-isomerase	cytoskeletal 9
Protein AMBP	Protein AMBP	Keratin, type I
Prothrombin	Bone marrow proteoglycan complement	cytoskeletal 10
Growth/differentiation factor 15	component C7	Keratin, type I
Insulin-like growth factor-binding	Neutrophil gelatinase-associated	cytoskeletal 14
protein 7	lipocalin	Histone H1.2
Cystatin-M	Serum albumin	Jupiter microtubule
Prostaglandin-H2 D-isomerase	Apolipoprotein D	associated
	Ribonuclease pancreatic	homolog 2
		Nucleoside
		diphosphate
		kinase A
		Keratin, type II
		cytoskeletal 1
		Trypsin-3

Table 3.
Proteins identified with highest scores in analyzed samples.

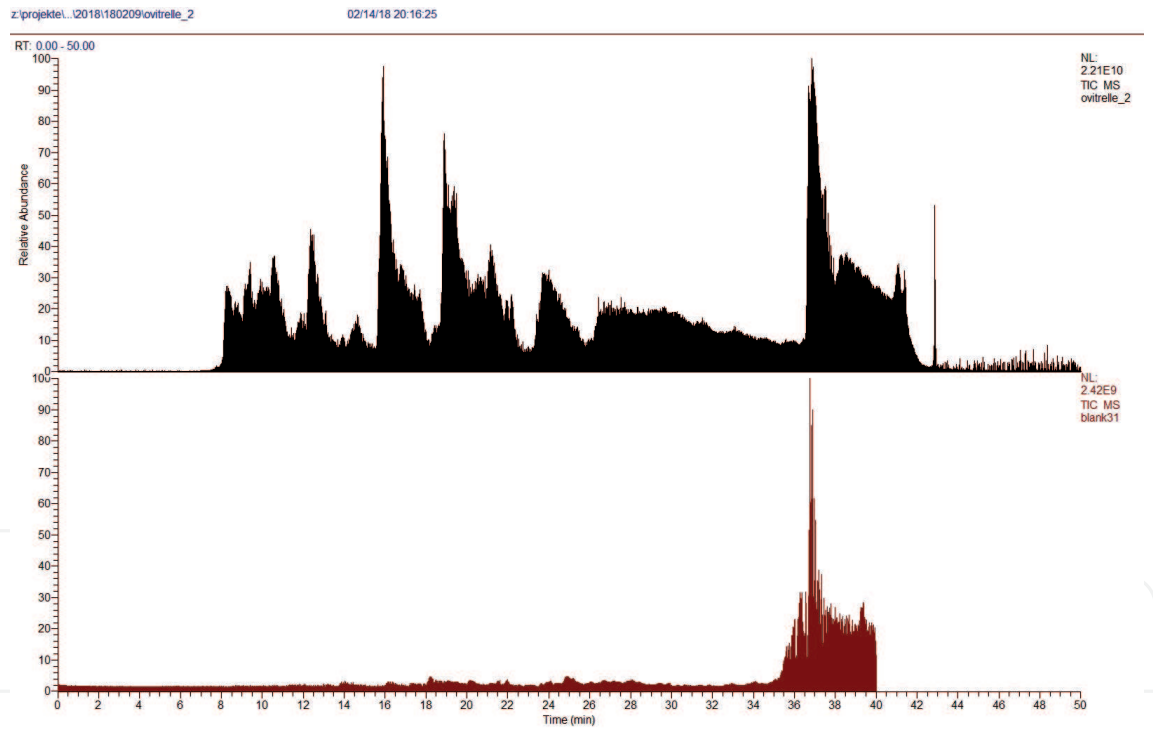


Figure 2.
Blank injection following analysis of digested recombinant hCG.

common contaminants such as keratins, in order to ensure that these proteins do not origin from previous injections of urinary formulations, blank sample injections (buffer used for dissolving peptides) were analyzed before and after the injection of each sample, and the TIC of such a blank injection is shown in **Figure 2**.

4. Discussion

In European Union, the production and the quality of medical products for both human and animal use is strongly regulated and controlled from both national

health agencies and qualified bodies and from the European Medicines Agency. Companies producing medicines and medical devices must follow strict and detailed guidelines and secure that all operations are performed under governing GMP and GLP rules. These precautions shall secure the quality of the product and the safe use for the patients. Thennati et al. has described the method for the quality control of the recombinant product is ensured through analytical steps using SDS-gel separation and MALDI-ToF analysis of the final product [17]. Therefore, it was a great surprise to identify a number of proteins originating from the starting product (urine) in different batches of the final product.

A discussion on contaminant proteins in hCG formulations has already been published [4, 5, 7, 17–19], and several publications address the possibility of the presence of harmful substances in commercial formulation [13–15, 20–22] but no final decision was made and the urinary-derived hCG formulations are still widely used although it has been shown that recombinant hCG can be used with the same success rate.

Due to the lack of published data and reports on hCG formulations and the lack of information about these products, we have analyzed commercially available formulations that are routinely prescribed for patients undergoing IVF treatment.

The major active component, hCG, was identified in all analyzed samples and, in addition, human serum albumin (HSA), luteotropin subunit beta (LSHB), and glycoprotein hormones alpha chain (CGA). The presence of HSA in all samples can be explained by its secretion in urine and by the need of growing CHO cells, for recombinant hCG production, in culturing medium supplemented with HSA, which might contain a number of other proteins that were not removed when HSA purification was performed. The pathway and interaction analysis, shown in **Figure 3**, explains the dependence of co-expression between the hCG, LHB, and CGA and explains why these proteins can also be identified in recombinant products. Obviously, the production of hCG also induces the expression of other proteins involved in ovarian steroidogenesis, hormone activity, and regulation of hormone levels, which are identified with high confidence with MS/MS analysis. However, in this case, the identification of LHB is a false positive one, and we cannot claim that this protein is really present in the recombinant sample. The reason is that hCG and LHB have a common amino acid sequence between amino acids on positions 22

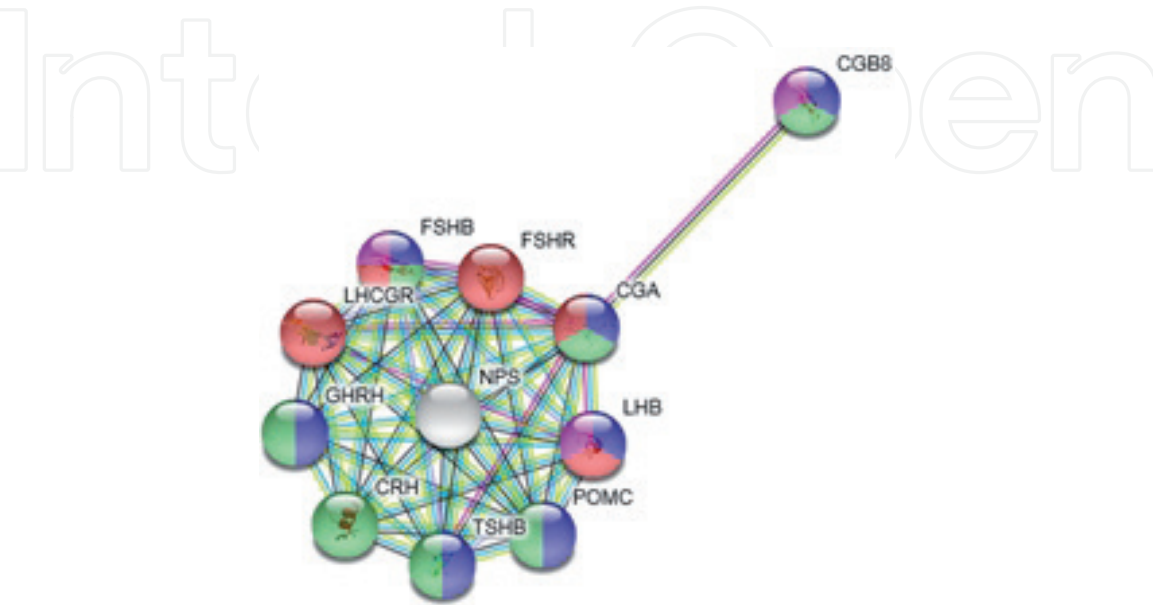


Figure 3. Pathway describing the interdependence of hCG and LHB both of which were identified in recombinant products.

and 131 in the protein backbone. If other parts of the sequence cannot be identified, we cannot tell the proteins apart. However, in this case, the identification score and the identification of other amino acids in the hCG sequence show an unique identification.

4.1 Contaminant proteins in urinary-derived samples

As for the contaminants in urinary-derived products, uromodulin, which is also the major urinary protein, was a major hit following the major component. However, other proteins, such as alpha-1-microglobulin or apolipoprotein D, and prostaglandin were identified with high confidence. The contaminants in urinary-derived hCG formulations resemble urinary proteins identified in urine-only samples, which were described in a recently published study on stress-induced urinary incontinence [23].

Keratin was one of the major alien contaminants, and its presence suggests a contamination during the sample production and the origin might be the insufficient air-conditioning or the contamination of the packaging units, which might be traced back to the use of latex-made gloves, dust containing skin particles, etc. We can exclude the contamination in our lab since all sample preparation steps have been performed using the laminar flow and using nitrile gloves. The blank samples show no identifications of keratin or of other contaminations.

Additional distinction between the urinary and the recombinant products can be seen when looking at the GO analysis of the biological processes where these proteins are involved, as shown in **Figure 4**.

4.2 Contaminant proteins in recombinant samples

Products originating from the production process employing recombinant method have significantly less contaminant proteins of human origin. To our best knowledge, until now, there are no reports describing contaminations for recombinant hCG. Upon analysis, the hCG was the major component identified with contaminants such as keratin and human serum albumin (HSA). These contaminants are easy to explain, we have given an explanation for keratin in previous section, and HSA is most probably a contaminant from the culturing medium of CHO that was not completely removed upon hCG extraction.

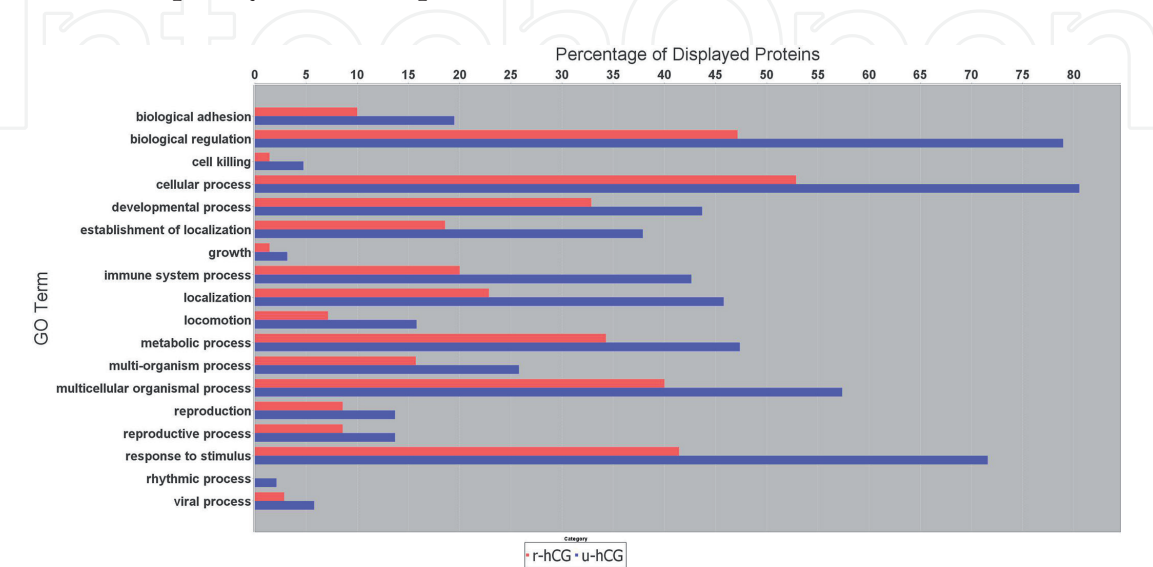


Figure 4. GO term analysis of proteins identified in both urinary and recombinant formulations showing differences in their abundancies in biological processes.

4.3 Embryogenesis and hCG, influence of contaminants

Often, hCG is also known as “the hormone of pregnancy”. It plays an important part for establishing and maintaining pregnancy, and it is extensively used in IVF procedures. A serial measurement of hCG every 48 hours is being used to confirm the early pregnancy and to distinguish between normally progressing pregnancies from ectopic pregnancies or spontaneous abortions [10]. It was shown that the miscarriage rate is higher when assisted reproductive techniques (ARTs) are applied than with naturally occurring pregnancy [11]. Girard et al. evaluated the association between early β -hCG concentration increases, blastocyst morphology, and pregnancy evolution in a single-blastocyst transfer program. In most IVF laboratories, blastocyst transfers are associated with higher success rates of implantation as compared with the cleavage stage embryo as shown by Oron et al. [12] and Girard et al. [10]. Most laboratories report that blastocysts are selected for transfer according to their morphological characteristics. That means that physicians decide on implantation based on, that is, expansion degree, hatching status of the blastocysts, inner cell mass, or trophectoderm characteristics.

It is taught that hCG is involved in embryo implantation by modulating the activity of collagenases and plasminogen activators in an in-vitro system, as described by Yagel et al. [13]. Furthermore, it is an interesting and noteworthy feature that most of the mediators that have earlier been considered essential for the implantation process (i.e., EGF and IL-1/IL-6) are also involved in the regulation of hCG biosynthesis by the placental syncytiotrophoblasts [13–15] probably by modulating trophoblast differentiation. Therefore, Licht et al. postulated that hCG may play a central part in the hypothetical embryo-maternal cross talk and tested the hypothesis by simulating the effect of a very early pregnancy on the decidualized endometrium. The results suggested that, no immunoreactive hCG was found in the peripheral circulation ($\text{hCG} < 5 \text{ mIU/mL}$) and the treatment did not alter progesterone secretion by the corpus luteum, thus suggesting that the effects observed were direct.

As described in previous paragraph, urinary-derived hCG contains high number of contaminant proteins. Considering that, particularly, EGF is present in high concentrations in almost all tested commercial preparations of urinary-derived hCG, and additional experiments are needed to find out whether the effects observed are due to hCG or to a contamination from the formulation.

Furthermore, contaminant proteins identified in hCG formulations can be the source of severe allergic reactions and can also trigger immune response that must not necessarily been manifested externally; however, it can negatively affect embryo implantation and embryogenesis. Koh et al. [16] and Phipps et.al [17] described the cases of IgE-mediated immunoreaction after intramuscular administration of urinary-derived gonadotropins. This type of allergic reactions occurs shortly after administration of urinary-derived gonadotropin, and it occurs with symptoms like shortness of breath, wheezing, flushing, general weakness, and dizziness. In all reported cases, the IgE-mediated allergic reaction could be confirmed after intradermal skin testing with the same uhCG lot. Testing with rhCG triggered no reaction, thus confirming the theory that some urinary proteins can trigger an immune response and might be responsible for anaphylactic reactions. Immune reaction could also influence the development of the embryo and hinder normal embryogenesis and embryo implantation in endometrium.

Kajihara et al. [18] proposed that some urinary-derived hCG (uhCG) impurities like EDN or ribonuclease 2 could influence inflammatory and immunological processes through regulation of apoptosis in endometrial cells and, therefore, directly influence embryo nesting and development during IVF. Considering that some patients undergo lengthy and intensive treatment with uhCG, an effect cannot be excluded.

Uromodulin, as the most abundant protein in human urine, is also detected in various uhCG formulations as the most abundant one. Uromodulin has been described as a powerful stimulator of the immune system through its ability to bind on the surface of almost all blood cells and to encourage the cellular production of cytokines, increase lymphocyte proliferation and phagocytosis [19, 20]. Using a mouse model, de Silva Antunes et al. suggested that uromodulin, but also some other urinary proteins, that is, major urinary proteins [13, 14, 17] or kidney androgen-regulated protein act as a possible allergens in the T-cell-mediated allergic reaction [21]. Otherwise, Phinuster et al. [22] suggested protective role of uromodulin in amniotic fluid in the fetus defense against anti-allogenic antibodies and immunosuppressive effect on T-cell-mediated allogenic rejection.

5. Conclusion

Formulations of hCG, both urinary and recombinant, contain contaminant proteins that originate from either starting material or has been introduced during the manufacturing process. The information about these contaminants cannot be identified on products' leaflets.

We could show that urinary-derived products contain a significant number of human proteins that obviously originate from the starting raw material—the human urine.

We think that this issue must be approached and discussed since the recombinant proteins show no such contamination and their use has been proven safe and effective.

Due to an important role played by hCG in embryo development in healthy subjects and embryo implantation during the IVF procedure, it is of great importance to thoroughly check the quality of the formulations and apply production steps for the best possible removal of all contaminant proteins from the final product and prevent possible adverse and allergic reactions, which might affect embryogenesis in general and embryo implantation in uterus.

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