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Breast levonorgestrel concentrations in women using a levonorgestrel-releasing intrauterine system☆·☆☆·☆☆☆

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ABSTRACT

Objective: To measure breast tissue and serum LNG concentrations in women using a LNG-IUS.
Study design: This pilot study was performed in 25 healthy women undergoing breast surgery at the Ghent University hospital. LNG concentrations were measured in serum and microdissected breast tissue samples using a validated ultra-performance liquid chromatography/tandem mass spectrometry assay.
Result(s): The mean LNG concentration in the 18 LNG-IUS users was 0.18±0.16 ng/mL in serum and 0.26±0.28 ng/g in breast tissue. For four women without any form of hormonal contraceptive (the negative controls), the mean concentrations were below the limit of quantification, i.e., 0.15 ng/mL and 0.20 ng/g, for serum and breast tissue, respectively. For the three positive controls the concentrations in the serum (20.5 and 3.4 ng/ml) and the breast (3.74 and 1.24 ng/g) were respectively for the 20 μg EE/100 μg users and 315 pg/ml in the serum and 1.17 ng/g in the breast for the minipill user. The intracellular free fraction of LNG may be as low as 0.008 ng/g.
Conclusion(s): The concentration of LNG in breast epithelium cells in women using the LNG-IUS is very low.
Implications: The relationship between the serum and breast tissue levels of LNG was studied in women using a LNG-IUS or oral LNG-containing contraception. Compared to oral contraception, the tissue levels of LNG in LNG-IUS users are much lower in the breast. It is not known what level of LNG exposure in the breast would stimulate *RANKL* and *WNT4* expression; such information is needed.

1. Introduction

The levonorgestrel (LNG)-releasing intrauterine system (LNG-IUS, Mirena®, Bayer, Germany) has been proven to be a very effective contraceptive. Leading organizations such as the Centers for Disease Control (CDC), World Health Organization (WHO), and National Institute for Health and Care Excellence (NICE) suggest that intrauterine systems could even be used as first-line contraception [1–3]. In many countries, an increasing number of women are currently using their fourth consecutive LNG-IUS and many even continue to use it after menopause for endometrial protection during estrogen replacement [4,5]. The LNG-IUS induces a high LNG concentration in the endometrium [6], which offers strong protection against hyperplasia. In contrast, the amount of LNG released into the systemic circulation is very low [7], but large individual variations have been described [8]. This systemic LNG release could be relevant for breast safety.

Some publications point out that during menopause, the use of systemic synthetic progestogens (progestins) is associated with an increased incidence of breast cancer [9,10]. Well-designed experimental studies indicated that some progestogens might interfere with apoptosis induced by estrogen [11]. Another study indicated that progesterone triggers the secretion of WNT4 (Wingless-related integration site4) and RANKL (receptor activator of nuclear factor-kappaB ligand). This signaling may increase the migratory potential of breast cells [12].

Currently, the long-term breast safety of LNG-IUS users is controversial. Some reports are reassuring [13,14], while others indicate an increase in breast cancer risk [15–17]. Although serum LNG concentrations have been reported in users of different types of LNG-IUS [18], data on actual LNG concentrations in the breast are currently lacking. Determination of these in vivo concentrations would allow in vitro experiments to be designed to study the influence of different

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LNG concentrations on different breast cancer lines. The aim of our study was to measure LNG concentrations in the breast tissue of LNG-IUS users that could be useful for planning future studies.

2. Materials and methods

2.1. Patients

LNG concentrations were determined in breast tissue and in serum of 25 healthy women undergoing reductive breast surgery for esthetic reasons. Normal histological findings in the recovered breast tissue were present in all women. Women with ductal hyperplasia were not included. A total of 18 women used the LNG-IUS. Their ages ranged from 17 to 54 years, and the BMI range was 22 to 29 kg/m^2. Four women who were not using any form of contraceptive served as negative controls. Two women using the 20 μg ethinyl estradiol (EE)/100 μg LNG combined oral contraceptive (COC) and one woman taking an oral LNG minipill (30 μg) served as positive controls. The contraceptive tablets were taken in the morning of the day prior to surgery by one patient (20 μg EE/100 μg LNG) and on the day of surgery by the two other patients (20 μg EE/100 μg LNG and 30 μg LNG). The study was approved by the UZ Gent ethical committee (EC 2005/022–10 022011). A written consent was obtained from all participating women.

2.2. Breast tissue and blood sampling

Breast tissue samples (100 mg) were immediately (snap) frozen in liquid nitrogen and stored at −80°C. The samples were kept frozen during manual, microscopic dissection into fractions. All fat tissue was removed, leaving for the most part only epithelium, blood vessels, extensive intercellular space with connective tissue and stromal cells, of which the proportions could not be determined. Blood samples were obtained prior to surgery and processed to obtain serum, which was also stored at −80°C.

2.3. Assays

The determination of LNG levels was performed by an in-house validated ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS, Xevo TQ-S, Waters, Milford, US) assay method. A gradient elution of acidified water (0.1% formic acid) and methanol was applied, and pumped at a constant flow rate of 0.3 mL/min onto an Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 μm, Waters).

To optimize the extraction procedure for both biological matrices, the protocol of Moser and coworkers was followed [19]. This method was properly validated following optimization of the extraction protocol. Validation of the assay comprised assessing precision (intra-lab reproducibility), linearity (R^2 of calibration line and lack-of-fit of 3 calibration lines), limit of detection (LOD) and limit of quantification (LOQ). The deuterated internal standard levonorgestrel-d6 was used to correct for procedural losses. Serum was spiked for precision evaluation at 0.1, 0.15 and 0.2 ng/mL LNG, and breast tissue was spiked at 0.2, 0.4 and 0.5 ng/g LNG. Relative standard deviations (RSDs) for precision of LNG analysis were 18.4, 18.1, and 9.8% in serum and 3.5, 1.1, and 4.2% in breast tissue, respectively. The LOD was 0.10 and 0.15 ng/g and the LOQ was 0.15 ng/mL and 0.2 ng/g in serum and breast tissue, respectively. Mean concentrations and standard deviations were calculated for the serum and breast tissue LNG measurements. When concentrations below the LOD were observed, they were set at LOD divided by 2. If concentrations between the LOD and LOQ were noted, the calculated value was assigned.

3. Results

The mean LNG concentration in LNG-IUS users was 0.18±0.16 ng/mL in serum and 0.26±0.28 ng/g in breast tissue. For the negative controls, the mean concentrations were below LOQs for both serum and breast tissue. For the 3 positive controls, the LNG concentrations were 20.5 and 3.37 ng/mL in serum and 3.74 and 1.24 ng/g in breast tissue for the 20 μg EE/100 μg LNG users; the corresponding concentrations in the minipill users were 0.31 ng/mL and 1.17 ng/g, respectively.

4. Discussion

We are the first to measure LNG concentrations in breast tissue of LNG-IUS users. Using a highly sensitive UPLC-MS/MS technique, our LODs were 0.10 ng/mL and 0.15 ng/g for serum and breast tissue, respectively. The serum LNG concentrations we found are in accordance with the levels (0.218 ng/mL) reported in the literature in LNG-IUS users [18].

A poor correlation was observed between the serum and breast tissue LNG concentrations in the LNG-IUS users. This may be due to the small sample size in our study and using data based on a single time point.

It is clear from our study that the breast tissue LNG concentrations in the women taking either the LNG COC or the LNG minipill are higher than the corresponding concentrations in the women using the LNG-IUS. However, the oral contraceptive concentrations are based on a single time point. To make a proper comparison of the LNG-IUS and the LNG oral contraceptives, it is essential to estimate the steady state LNG values over a 24-h period from the oral contraceptives. Use of a single time point is of very limited value, and is a limitation of our study.

The available LNG data for the positive controls show that the serum concentrations were 20.5 and 3.3 ng/mL in the COC users, whereas the concentration in the minipill user was 0.31 ng/mL. The COC user who had an LNG concentration of 20 ng/mL took the pill on the morning of the day prior to surgery. The corresponding breast tissue LNG concentration in this woman was 4 ng/g. Both the serum and tissue LNG levels appear to very high and the serum value is significantly higher than values previously reported in literature [20,21]. As no analytical artifacts were noted and this concentration confirmed repeatedly, we hypothesize that this may be explained by other medication administered and/or procedures performed to the patient prior to surgery.

In our study, we measured total LNG levels, of which approximately 97% are protein bound (sex hormone binding globulin (SHBG)-bound, 47%; albumin-bound, 50%; unbound, 3%) [22]. The unbound LNG is hypothesized to be available for biologic activity in target cells as well as prone to metabolism. However, it is possible that albumin-bound LNG, being loosely bound, could dissociate in tissue capillaries, enter the cell, and become bioavailable [23].

The circulating unbound LNG, like other steroids, can diffuse freely through cell membranes and intercellular spaces [24]. The LNG concentration in the breast tissue obtained during our UPLC-MS/MS quantification is indeed total LNG, but the proportions that are protein-bound and unbound are not known. During processing of the tissue the hydrogen bonding involved in the binding of steroids by SHBG and albumin are destroyed. SHBG circulates as a homodimer. Each SHBG monomer contains 2 domains at the N-terminal end of the protein that enable the binding of sex steroids. The serine residue within this binding pocket is important in androgen and estrogen binding and forms hydrogen bonds with the ketone group at the C-3 position of certain androgens such as testosterone and of progestins such as LNG. The hydrogen bonds are about 10 times weaker than covalent bonds. This degree of binding strength allows for spontaneous dissociation of the steroid. The rate of dissociation plays a key role in determining how much of the steroid enters target cells for binding to receptors.

Albumin is quantitatively the most abundant protein in the circulation and accounts for about 60% of the total serum protein content. It

has a very high capacity for binding steroids, to which it binds with low but different affinities. Albumin consists of 3 domains, which have binding pockets formed predominantly of hydrophobic and positively charged amino acid residues. It is expected that LNG has a similar association constant (Ka) for albumin as testosterone ($2–4\times10^4$ L/mol with a very fast dissociation of about 1 s) [23]. The Ka of LNG for SHBG has been reported to be 9.1×10^6 L/mol [25] compared to around 1×10^9 L/mol for testosterone [23].

During tissue processing, we used microscopic dissection of the surgically removed breast tissue. All fat tissue was removed, leaving for the most part only epithelium.

The breast tissue investigated in this study consisted of breast epithelium cells, glandular lumen, blood vessels, and an extensive intercellular space with connective tissue and stromal cells. Albumin and possibly SHBG can diffuse through the vessel wall into the intercellular space. It may be assumed that the some of the LNG in intercellular space is also bound to these binding proteins [26]. Since it is generally accepted that <3% of circulating LNG is not bound to albumin or to SHBG, the actual biologically available LNG in the breast epithelium cells, which are the susceptible cells for breast cancer, may be substantially lower. If only 3% of the measured LNG in our breast tissue diffuses into the cell then the final intracellular LNG would be 0.008 ng/g. Whether this low concentration in the breast is sufficient to activate the *RANKL* and/or *WNT4* signaling system remains to be established.

In vitro studies have indicated possible mechanisms of action of LNG on breast cancer cell lines, through 17β-hydroxysteroid dehydrogenases (17β-HSDs). LNG has a potent effect on 17β-HSD1 and 17β-HSD2 in T47D cells. In T47D cells LNG upregulates the expression of the reductive 17β-HSD1 and downregulates the expression of the oxidative 17β-HSD2. These changes in enzyme expression lead to more bioactive estrogen, which indicates that LNG can indirectly influence ER activity [27,28]. At the LNG concentrations detected in breast tissue in our study, no influence on 17β-HSD1 and 17β-HSD2 was observed in highly sensitive T47D cells in ZR75–1 and MCF-7 cell lines.

LNG may also enhance VEGF production. VEGF is an important factor in the proliferation and migration of vascular endothelial cells (i.e., angiogenesis). It is an essential step for tumor growth, expansion and metastasis. Again, at the LNG concentrations observed in the breast in our study, no increases in VEGF mRNA expression has been described in T47D cells [29]. Nevertheless, until studies have been performed at these low LNG concentrations, LNG may still influence breast cells or breast cancer cells that may be present subclinically.

In conclusion, low LNG concentrations were detected in breast tissue of women using the LNG-IUS. The observed LNG concentrations offer a solid basis for further research regarding the influence of LNG on the breast.

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