ALÉXIA HERMANNY

LEVONORGESTREL COMO CONTRACEPTIVO DE EMERGÊNCIA E SUA INFLUÊNCIA SOBRE ALGUMAS FUNÇÕES ESPERMÁTICAS

Tese de Doutorado

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Errata do nome da aluna:

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Prof. Dr. José Barreto Campelo Carvalheira
Coordenador de Comissão de Pos-Graduação
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Dedico este trabalho...

... Aos meus pais,
por terem me dado, acima de tudo, a VIDA.

... À minha mãe querida,
que sempre me guiou pelo melhor caminho e,
mesmo com a saudade e a distância,
ainda assim continua viva e presente em minha vida.

... Ao meu pai,
que sempre me apoiou e compreendeu a minha ausência,
mesmo quando minha presença poderia lhe trazer companhia e
conforto nos momentos difíceis que passamos.

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" A ciência humana de maneira nenhuma
nega a existência de Deus.
Quando considero quantas e quão
maravilhosas coisas o homem compreende,
pesquisa e consegue realizar,
então reconheço claramente que
o espírito humano é obra de Deus,
e a mais notável."

(Galileu Galilei)

Sumário

Símbolos, Siglas e Abreviaturas

AE – Anticoncepção de emergência

AR – *Acrossomal reaction*

EC – *Emergency contraception*

LH – Hormônio luteinizante

LNG – Levonorgestrel

OMS – Organização Mundial da Saúde

P – Progesterona; *Progesterone*

RA – Reação acrossomal

SIU-LNG – Sistema intrauterino liberador de LNG

UNICAMP – Universidade Estadual de Campinas

WHO – *World Health Organization*

Resumo

O mecanismo de ação do levonorgestrel (LNG) na anticoncepção de emergência (AE) ainda não está totalmente esclarecido e seu efeito nas funções espermáticas também não está explicado. Os objetivos deste trabalho foram avaliar se o LNG, em dose igual à observada após a ingestão oral para AE, poderia afetar espermatozoides expostos *in vitro* à tuba uterina humana e realizar uma revisão bibliográfica sobre o efeito do LNG nas diferentes funções espermáticas. Foram realizados 15 experimentos. As tubas uterinas foram removidas através de mini-laparotomias e foram perfundidas com uma suspensão contendo $1x10^6$ espermatozoides móveis, com e sem LNG. A tuba correspondente ao lado onde o folículo dominante estava presente recebeu a suspensão com LNG em pacientes alternados. Após um período de incubação de 4 horas, o istmo e a ampola de cada tuba foram separados. Cada segmento foi lavado separadamente e o material obtido foi avaliado quanto ao número de espermatozoides móveis recuperados, número de espermatozoides aderidos ao epitélio tubário e taxa de reação acrossômica (RA). A presença do LNG não afetou significativamente o número de espermatozoides móveis recuperados do istmo e da ampola, e não afetou o número de espermatozoides aderidos ao epitélio tubário. O LNG

também não influenciou a taxa de RA. Diferenças significativas também não foram observadas quando o lado ovulatório foi considerado. A revisão bibliográfica realizada deixou evidente que existem poucos estudos que analisam a influência do LNG como AE sobre as funções espermáticas, apesar de este ser um possível mecanismo de ação. Além disso, os estudos revisados utilizam diferentes métodos de avaliação e os resultados são, muitas vezes, contraditórios. De acordo com os resultados observados na literatura, quando o LNG é usado na AE, provavelmente não atinge concentração plasmática suficiente para ser reconhecido pelos receptores de progesterona (P). Resultados positivos só foram observados quando a dose de LNG utilizada nos experimentos foi comparável ao sistema intrauterino liberador de LNG (SIU-LNG), ou seja, muito maior que a utilizada na AE. O LNG, em dose similar à observada no plasma após a ingestão oral para AE, não afetou o número, a aderência ao epitélio tubário, a distribuição e a taxa de RA de espermatozoides na tuba uterina humana, *in vitro*. De acordo com os resultados observados na literatura, se o LNG, na concentração utilizada para AE, afeta ou não a função espermática ainda não está claro, e mais estudos são necessários.

Palavras-chave: anticoncepção de emergência; levonorgestrel; função espermática; tubas uterinas; reação acrossômica; espermatozoide humano.

Summary

The mechanism of action of levonorgestrel (LNG) as emergency contraception (EC) is still under debate and the effect upon sperm function is partially explained. The aim of this study was to assess if LNG in a similar dose to those observed in serum after oral intake for EC could affect the spermatozoa when exposed *in vitro* to human tubes and also to give an overview of the effect of LNG as EC on several sperm functions. Fifteen mini-laparotomies were performed, the ovulatory side was recorded and both tubes were removed and perfused with a suspension of $1x10^6$ of motile spermatozoa, one with LNG and the other without it. After an incubation period of 4hours the tubes were cut to separate the isthmus and the ampulla. Each segment was flushed and the material was evaluated regarding the motile sperm number, the number of spermatozoa adhering to the oviductal epithelium and acrosome reaction (AR) rate. The addition of LNG did not significantly affect the number of recovered spermatozoa neither at the isthmus nor at the ampulla or the number of recovered spermatozoa adhered at the human tubal epithelium. Additionally, LNG did not influence the rate of AR. There were no significant differences even when the ovulatory side was taken into account. The present review showed that there are

few studies which focus on the influence of LNG as EC upon sperm functions; albeit it is a plausible mechanism of action. Additionally, the different studies used different methods of evaluation and the results were in many cases contradictories. According to the results observed at the literature, when LNG is used as EC, it is probable that the drug does not achieved sufficient serum concentrations in order to be recognized by the progesterone (P) receptors. Positive results only were observed when the dose of LNG used in the experiments was much higher (comparable to the LNG-IUS) than the proposed for EC. LNG in a similar dose to that observed in serum after oral intake for EC did not affect the number, the adhesion to tubal epithelium, distribution, and AR rate of spermatozoa at the human Fallopian tubes *in vitro.* According to the results observed at the literature, if the LNG in doses used for EC, affects sperm function or not, it is still uncertain and warrants further studies.

Key-words: emergency contraception; levonorgestrel; sperm function; Fallopian tubes; acrosome reaction; human spermatozoa.

1. Introdução

A anticoncepção de emergência (AE) é um método contraceptivo ocasional utilizado com o objetivo de evitar a gravidez em mulheres que tiveram coito sem proteção anticoncepcional, que sofreram abuso sexual, ou por mulheres que possuem razões para acreditar em uma eventual falha do método anticoncepcional adotado (1).

Entre os métodos hormonais, os mais utilizados na AE são o regime de Yuzpe e o regime com levonorgestrel (LNG) puro. O regime de Yuzpe consiste em duas doses da combinação de 100µg de etinilestradiol e 500µg de LNG, ingeridas com intervalo de 12 horas, sendo que a primeira dose deve ser tomada até 72 horas após o coito desprotegido. No regime com LNG puro, o progestágeno é administrado oralmente na forma de dois comprimidos de 0,75mg cada um, ingeridos em intervalo de 12 horas, e até as 72 horas seguintes ao coito não protegido (2). Atualmente a administração de uma única dose de 1,5mg até 72 horas após o coito desprotegido é o tratamento mais aceito para AE, por ser mais simples e não apresentar qualquer diferença na eficácia ou aumento nos efeitos

colaterais (3; 4). A concentração plasmática de LNG após o tratamento para AE varia de 7,9ng/ml a 12,3ng/ml para a ingestão de duas doses de 0,75mg, com 12 horas de intervalo, ou para dose única de 1,5mg, respectivamente (5).

Um estudo desenvolvido pela Organização Mundial da Saúde (OMS) comparou a eficiência do regime de Yuzpe com o LNG puro e mostrou que o LNG foi mais eficaz, evitando um maior número de gravidezes. Neste estudo também ficou demonstrado que a eficácia de ambos os tratamentos foi maior quanto mais próximo do coito foram administrados (3).

Embora o LNG seja largamente utilizado como AE, o seu exato mecanismo de ação ainda não está totalmente claro e por isso tem sido alvo de extensas discussões (3; 6; 7). Entretanto, já está estabelecido que sua eficiência depende da fase do ciclo menstrual em que a mulher se encontra, e aumenta quanto mais próximo ao coito desprotegido for administrado (1; 8). Os mecanismos de ação mais aceitos referem-se às alterações no pico do hormônio luteinizante (LH), na ovulação, no desenvolvimento folicular e do corpo lúteo, influência na penetração e migração espermática e interferência no processo de fertilização (6; 9; 10; 11). Também já foi demonstrado que o LNG só é capaz de impedir a gravidez se a fertilização ainda não tiver ocorrido.

Em mulheres foi mostrado que o período fértil do ciclo menstrual é constituído por seis dias, que incluem os cinco dias que precedem à ovulação, e o próprio dia da ovulação (12). No momento do coito os espermatozoides são depositados na vagina e permanecem nas criptas cervicais por horas ou até

mesmo dias, antes de percorrerem o caminho em direção às tubas uterinas (13). Consequentemente, pode-se levantar a hipótese de que os espermatozoides depositados na vagina de mulheres que utilizarem o LNG para AE poderão ser expostos a concentrações desconhecidas do progestágeno e sofrerem alguma interferência na sua capacidade fertilizante. Recentemente Noé e colaboradores (14) mostraram que mulheres que receberam LNG para AE durante o período fértil, mas antes da ovulação, não engravidaram, apesar da evidência de ovulação após o tratamento. Este resultado sugere que outro mecanismo, que não a supressão da ovulação, impede a gravidez quando o LNG é usado na AE por mulheres no período fértil.

Contudo, a possibilidade de um dos mecanismos de ação do LNG ser o de agir sobre o espermatozoide e assim exercer seu efeito contraceptivo tem sido pouco estudada e ainda permanece sem conclusão (15; 16; 17; 18).

O trabalho de Kesseru e colaboradores (15) mostrou que o LNG, quando usado como AE, poderia afetar os espermatozoides e a migração espermática. Foi observado que uma única dose oral de 0,4mg de LNG, 3 a 10 horas após o coito, diminuiu o número de espermatozoides recuperados da cavidade uterina, provocou alcalinização do fluido intrauterino, imobilizando os espermatozoides, e aumentou a viscosidade do muco cervical, prejudicando a passagem de espermatozoides para o interior do útero. Este trabalho é citado em vários estudos que abordaram o mecanismo de ação do LNG usado na AE; entretanto, as técnicas utilizadas há mais de 30 anos possivelmente estão ultrapassadas.

Por isso novos estudos foram realizados, a maioria por este grupo, utilizando técnicas diferentes, mais modernas e que abordam outros aspectos da função espermática, como reação acrossômica (RA), ligação à zona pelúcida (ZP), receptores para d-manose, aderência de espermatozoides ao epitélio tubário, entre outros. Os trabalhos mais recentes, com metodologias mais modernas e atuais, não observaram efeito do LNG usado na AE sobre a função espermática e nenhum deles conseguiu repetir os resultados obtidos por Kesseru e colaboradores (15).

Com base no conhecimento de que a ocorrência da RA é um dos processos-chave na fertilização e de que a progesterona (P) natural tem efeito direto nos espermatozoides, inclusive como promotora da RA, Bahamondes e colaboradores (17) avaliaram *in vitro* o efeito de diferentes concentrações de LNG na taxa de RA de espermatozoides humanos capacitados. Observaram que altas concentrações de LNG (200ng/ml a 800ng/ml), compatíveis com aquelas observadas no fluido uterino de usuárias de SIU-LNG, foram capazes de induzir a RA. Em outro estudo, espermatozoides capacitados foram expostos a concentrações de 1.000ng/ml e 10.000ng/ml de LNG e os resultados também mostraram que o LNG, nas concentrações utilizadas, aumentou significativamente a proporção de células com acrossoma reacionado em relação aos controles (19).

A maioria dos espermatozoides que migram para as tubas uterinas possui o acrossoma intacto e somente alguns poucos sofrem RA espontânea (20). Considerando que a RA é um evento que acontece quando o espermatozoide entra em contato com a superfície do oócito (21), pode-se supor que a indução

precoce da RA por substâncias específicas, como o LNG, diminuiria a quantidade de espermatozoides com potencial para fertilizar.

Porém, quando espermatozoides capacitados foram expostos *in vitro* a concentrações de LNG similares aos níveis séricos observados após a administração oral para AE (5), não foi observada qualquer alteração na taxa de espermatozoides reacionados (22).

Outro trabalho mostrou que espermatozoides humanos tratados *in vitro* com doses de LNG dez vezes menor (1ng/ml), igual (10ng/ml) e dez vezes maior (100ng/ml) que os níveis séricos observados após a administração oral para AE, apresentaram comprometimento da motilidade já com 10ng/ml, porém nenhum efeito foi observado na RA (18).

A administração de 1,5mg de LNG em mulheres laqueadas, após diferentes tempos pós-coito, não afetou a qualidade do muco cervical, nem a penetração dos espermatozoides, expressa como o número de espermatozoides recuperados da cavidade uterina, e nem sua capacidade fertilizante, mostrada através da reação acrossomal (RA) (23).

A presença de espermatozoides em número adequado na tuba uterina não é suficiente para garantir a fertilização. A adesão dos espermatozoides ao epitélio tubário tem sido apontada como um importante pré-requisito para a fertilização *in vivo* em várias espécies (24). O contato entre espermatozoide e endossalpinge de alguma maneira protege o espermatozoide durante a permanência nas tubas. *In vitro,* a fertilidade e a motilidade espermáticas são mantidas por mais tempo

quando os espermatozoides são incubados com epitélio da endossalpinge (25). Diferentemente do que ocorre em outras espécies, um reservatório espermático distinto não foi observado nas tubas humanas, mas, um reservatório funcional é estabelecido e foi mostrado que, *in vitro*, os espermatozoides permanecem nos istmos, aderidos ao epitélio tubário, embora de forma intermitente (26; 13). A migração espermática para os ovidutos e através destes, e a sua adesão ao epitélio tubário, mecanismo necessário para alcançar a fertilização, são controlados pelos esteróides sexuais (27).

Espermatozoides humanos foram incubados com explantes de istmo e ampola de tuba em meio de cultura com e sem LNG, e foi observado que na presença do LNG o número de espermatozoides aderidos tanto ao istmo como à ampola foi significativamente menor (28). Estes resultados deram embasamento para a suposição de que o LNG poderia afetar a interação entre espermatozoide e epitélio tubário e consequentemente algumas funções espermáticas.

O mecanismo de ação do LNG na AE ainda não está totalmente esclarecido e pouco foi explorado nas tubas uterinas, onde ocorre a fertilização. Por isso, entendeu-se ser necessário o estudo do efeito do LNG, como AE, sobre os espermatozoides presentes na tuba uterina de mulheres, após a perfusão de solução espermática seguida de incubação das tubas, com e sem LNG, para avaliar o efeito do LNG sobre o número de espermatozoides móveis recuperados, o número de espermatozoides aderidos em cada segmento tubário e também a taxa de RA.

2. Objetivos

2.1. Objetivo Geral

Avaliar se o LNG, em dose igual à observada após a ingestão oral para AE, poderia afetar espermatozoides recuperados *in vitro* de tubas uterinas após perfusão das mesmas com solução espermática, com e sem LNG, seguida de incubação em meio de cultura, também com e sem LNG, e realizar uma revisão bibliográfica sobre o efeito do LNG sobre as diferentes funções espermáticas.

2.2. Objetivos Específicos

- Avaliar o número de espermatozoides móveis recuperados do istmo e da ampola após incubação de tubas uterinas com e sem LNG.
- Avaliar o número de espermatozoides aderidos ao istmo e à ampola após incubação de tubas uterinas com e sem LNG.

- Avaliar a taxa de reação acrossomal nos espermatozoides recuperados do istmo e da ampola após incubação com e sem LNG.

- Avaliar se os resultados observados foram diferentes em função da presença ou ausência de folículo.

- Revisar o efeito do LNG como AE sobre diferentes funções espermáticas.

3. Publicações

Artigo 1 – **In vitro assessment of some sperm functions exposed to levonorgestrel in human tubes**

Alexia Hermanny, M. Valeria Bahamondes, Francisco Fazano, Nadia M. Marchi, Maria Elena Ortiz, Maria Heloisa RR Genghini, Horacio B. Croxatto, Luis Bahamondes

Artigo enviado para *Reproductive Biology and Endocrinology Journal*

Artigo 2 – **The effect of levonorgestrel as emergency contraception on spermatozoa function: a review**

Alexia Hermanny, Josiane de Nascimento, Francisco Fazano, Maria José Munuce, Luis Bahamondes

Artigo enviado para *Fertility and Sterility*

3.1. Artigo 1

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In vitro assessment of some sperm functions exposed to levonorgestrel in human tubes

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Abstract

Background: The mechanism of action of levonorgestrel (LNG) as emergency contraception (EC) is still under debate and the effect upon sperm function is partially explained. The aim of this study was to assess if LNG in a similar dose to those observed in serum after oral intake for EC could affect the spermatozoa when exposed *in vitro* to human tubes. **Methods:** Fifteen mini-laparotomies were performed, the ovulatory side was recorded and both tubes were removed and perfused with a suspension containing $1x10^6$ of motile spermatozoa, with LNG or not. After an incubation period of 4h the tubes were cut to separate the isthmus and the ampulla. Each segment was flushed and the material was evaluated regarding the motile sperm number, the number of spermatozoa adhering to the oviductal epithelium and acrosome reaction (AR) rate. **Results:** The addition of LNG did not significantly affect the number of recovered spermatozoa neither at the isthmus nor at the ampulla or the number of recovered spermatozoa adhered at the human tubal epithelium. Additionally, LNG did not influence the rate of AR. There were no significant differences even when the ovulatory side was taken into account. **Conclusions:** LNG in a similar dose to that observed in serum after oral intake for EC did not affect the number, the adhesion to tubal epithelium, distribution, and AR rate of spermatozoa at the human Fallopian tubes *in vitro.* The possible effect of LNG, as EC, upon sperm functions remains poorly understood.
Key-words: emergency contraception; levonorgestrel; acrosome reaction; Fallopian tubes, human spermatozoa.

Background

Levonorgestrel (LNG) is a progestin used in emergency contraception (EC) and currently two 0.75 mg pills taken 12 hours apart or one dose of 1.5 mg up to 72 h after unprotected sexual intercourse is the recommended dose [1,2]. Despite the wide use of LNG as EC worldwide, the mechanism of action is still under debate and probably involved several mechanisms which are dependent on the time of administration in relation to sexual intercourse and the phase of the menstrual cycle in which it is taken [3,4].

One of the proposed mechanisms of action is the effect of LNG upon spermatozoa and their functions [5-8]. However, this effect is still poorly understood. Progesterone (P) is the promoter of alterations of sperm functions related to fertilization like capacitation, sperm hyperactivation and acrossomal reaction (AR) [9-15]. Albeit LNG has a weak agonist effect upon the P sperm receptors, it was observed [8] that high LNG concentrations (200 to 800 ng/mL) were capable to induce AR *in vitro* in human spermatozoa. However, when the spermatozoa were exposed to LNG concentrations similar to those observed in serum after intake for EC [16] no effect was observed [17]. Additionally, when human spermatozoa were treated *in vitro* with LNG at doses of 1 ng/mL, 10 ng/mL and 100 ng/mL which represents lower, similar and higher levels than those observed in serum after LNG intake for EC, it was observed motility impairment with the dose of 10 ng/mL; however, no effect was observed in the AR [7]. Otherwise, administration of 1.5 mg of LNG to sterilized women at different times after coitus did not affect the quality of cervical mucus, the sperm penetration to uterine cavity or AR [18].

After ejaculation mammalian spermatozoa are not able to fertilize the oocyte and this capacity is acquired as a consequence of a series of physiological and functional alterations called capacitation. Sperm capacitation occurs during sperm migration in the female genital tract [19]; however, it was noted that the adhesion of spermatozoa to the tubal epithelium is an important condition to *in vivo* fertilization in several species [20]. The interaction between spermatozoa and the endosalpinx offers some protection to spermatozoa and *in vitro* it was observed longer sperm fertility and motility when spermatozoa were incubated with tubal epithelium [21]. Spermatozoa present two modifications when they are in preparation for fertilization: capacitation and hyperactivation. Capacitation includes changes at the plasmatic membrane including the layer of protein and several lipids which prepare the cells to the AR and fertilization [22]. Those spermatozoa which complete AR prematurely are unable to penetrate the ZP because they loose the enzymatic acrosomal content [10,23].

Due to the fact that the effect of LNG as EC upon sperm function is still poorly understood and as far as we know this effect was not tested at the site of fertilization, the objective of this study was to assess if LNG in a similar dose to those observed in serum after oral intake for EC could affect the spermatozoa when exposed *in vitro* to human tubes.

Methods

The study was conducted at the Human Reproduction Unit, Department of Obstetrics and Gynaecology, School of Medicine, University of Campinas (UNICAMP), Campinas, Brazil. All the women and semen donors gave their

written informed consent and the study protocol was approved by the Institutional Ethical Committee. Women aged 25–41 years old were invited to participate in the study. The admission criteria included women who required surgical sterilization, who had indication of abdominal route of the surgery, with regular menstrual periods (25–35 day intervals), without any knowledge about tubal diseases, neither hormonal nor intrauterine contraceptives use during the cycle of the experiment, no use of any other hormone therapy, no breast feeding or pregnancy in the 6 months preceding the study.

2.1. Experiments

Women were instructed to use condom as a contraceptive method from the first day of the cycle through the day of the surgery to avoid unplanned pregnancy. In all women, follicular development was monitored daily since day 8^{th} of the menstrual cycle by ultrasound using a 5.0 MHz vaginal probe (Justavision 400, Toshiba, Toshigi-Ken, Japan). When the dominant follicle reached a diameter between 14 and 17 mm a surgical sterilization was schedule for the next day. All experiments were performed before ovulation to avoid the influence of the P milieu upon AR and possible confounding effect of P on sperm adhesion to tubal epithelium. To confirm the follicular phase of menstrual cycle event, a blood sample was collected on the day of the surgery and the serum was separated. The samples of serum were stored at −20°C until the measurements. Presence of the follicular phase of the cycle was confirmed by serum P levels below 3 ng/mL [24]. The determination of P was done in duplicate using a commercial electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim,

Germany) with a measurement range of 0.030–60.0 ng/ml and an inter-assay coefficient of variation (CV) of 2.4% and an intra-assay CV of 2.7%.

A mini-laparotomy was performed in all women under anesthesia. Both tubes were removed gently from the proximal portion. The side of the dominant follicle was identified and recorded. The tubes were put in two separate Petri dishes with HEPES-buffered modified Human Tubal Fluid medium (HTF-HEPES; Irvine Scientific, Santa Ana, CA, USA) and transferred immediately to the laboratory and the excess of tissue (mesosalpinx) was removed.

2.2. Semen samples

Fifteen semen samples were obtained from healthy donors with normal sperm analysis criteria according to the World Health Organization Manual [25]. Semen was collected by masturbation in sterile plastic jars after 3 days of sexual abstinence. After evaluation, the sample was divided in two tubes with equal volume. Motile spermatozoa of each fraction were selected by a swim-up technique using 1 mL of HTF-HEPES medium supplemented with 35 mg/mL of bovine serum albumin (BSA), 1 nM of estradiol (E_2) and 25 nM of LNG (0.1% from a solution with 25 µM of LNG in ethanol) in one and in the other instead of LNG the same concentration of ethanol present in the LNG treated sample was added. After 1 h of incubation the supernatant with the motile spermatozoa was removed carefully and a sample was placed into a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) heated at 37ºC and examined under a microscope. After evaluation each fraction was resuspended in the same medium in

order to have a sperm concentration of $10x10^6$ motile spermatozoa/mL. A sample of each sperm suspension was taken to evaluate the AR as described below.

2.3. Fallopian tube perfusion

One of the tubes was perfused with a non-sharp needle from the proximal portion with 100 µL of sperm suspension containing $1x10^6$ motile spermatozoa with the medium described above with the LNG and in the other tube the LNG of the sperm suspension was substituted by the same concentration of ethanol present in the LNG treated sample. The tube corresponding to the ovary with a dominant follicle received the suspension with LNG and the other tube with the vehicle. The side of tube (dominant follicle vs. non dominant follicle) that received LNG alternated for each woman ipsilateral or contralateral, in regard of the dominant follicle. The perfusion was done very slow to avoid that the solution spilled outside the tube. The procedure was done outside the culture medium over a glass with controlled temperature. After perfusion with the sperm suspension, the tubes were incubated separately at 37°C during 4 h in a Petri dish containing the same medium used in the sperm suspension to allow capacitation.

2.4. Fallopian tube processing

After incubation, the tubes were cut to separate the isthmus and the ampulla and each segment was flushed twice, the first time with 5 ml of HTF-HEPES medium and the second with an equal volume of phosphate-buffered saline (PBS; GIBCO, BRL, Life Technologies, Inc, Grand island, NY, USA) medium containing 0.5% Triton-X100 (Sigma-Aldrich, St Louis, Missouri, USA), a nonionic surfactant, in order

to remove the spermatozoa adhering to the oviductal epithelium [26,27]. The flushing materials from the tubal segments were centrifuged and the pellets were resuspended in 100 µL of PBS. The materials from the first flushing with HTF-HEPES medium were evaluated to assess motile sperm number and AR rate. Only sperm number was evaluated in the flushing materials obtained with Triton-X100 because all the sperm cells died when contacted with Triton-X100 medium. The culture medium from the Petri dish in which the tubes were immersed during incubation was transferred to test tubes and centrifuged and the pellets were also diluted in 100 µL of PBS for further evaluation of sperm number, motility and AR rate.

The samples after Triton flushing were evaluated in a Neubauer chamber to verify the number of recovered spermatozoa in each segment. The other samples were divided to allow that 50 µL were used to count the number of motile spermatozoa in a Mackler counting chamber and other 50 µL were used to Hoechst stain. To the 50 µL of the washing medium with the recovered spermatozoa was added 50 µL of Hoechst 33258 (bis-Benzimide; Sigma Chemical Co; St. Louis USA, B2883) at 1 µg/mL. The mixture was incubated during 5 min at 37ºC and after that two times washing were performed to remove the stain excess with PBS and centrifugation. The pellets were re-suspended in 50 µL of PBS. Two slides of each washing were prepared and allowed to dry in a dark ambient at room temperature.

2.5. Assessment of AR status

The fluorescent probe fluorescein isothiocyanate-labelled *Pisum sativum* lectin (FITC-PSA; Sigma-Aldrich) was used to evaluate the AR status. The slides prepared from the spermatozoa suspension stained with Hoechst, after leaving

them to dry were immersed in cool absolute methanol at -20ºC for 30 s. After that, the slides were stained by immersing them in FITC-PSA at a concentration of 40 µg/mL in PBS for 30 min and protected from light at room temperature. After incubation, the slides were washed in PBS and stored in the dark until evaluation for AR and vitality. Evaluation was done using a fluorescent microscope (Zeiss, Axioplan II, Jena, Germany) equipped with a specific filter for the FITC-PSA method (494-blue excitation; 520 emission; 510–514 barrier) and with a filter for the Hoechst 33258 stain (343-UV excitation; 480 emission; 400 barrier). Two hundred cells were evaluated in the fields chosen at random. The sperm viability was assessed only because habitually dead spermatozoa presented acrosomal reaction and consequently it was used only to discriminate dead or alive spermatozoa and consequently which cells were necessary to evaluate an acrosomal reaction.

The spermatozoa that were considered acrosome-reacted were those with the following patterns: (i) patchy fluorescence of the acrosomal region (partially acrosome reacted) and (ii) fluorescence of the equatorial band only (acrosome reacted) [28]. The AR rate considered was the difference between the rate observed in the perfusion material before incubation and the rate observed in sperm suspension obtained after segment flushing.

2.6. Statistical analysis

The software SAS 9.2 was used to analyze the data. The mean number of recovered spermatozoa and AR rate with LNG or not were compared by Mann-Whitney test. The level of significance was established at $p < 0.05$ and all values are shown as mean ± standard error of the mean (SEM).

Results

Fifteen experiments were conducted. However, two women showed P levels > 3 ng/mL on the day of the experiment and were excluded from the analysis to avoid the influence of P on acrosomal reaction. Most of the spermatozoa recovered after the first tubal flushing was motile. The total number of spermatozoa was similar between the isthmus with or without the addition of LNG. Nevertheless the number of recovered spermatozoa was almost 10 times greater at the ampulla than at the isthmus; however, also with no differences when LNG was or not added to the medium. When the recovered number of spermatozoa was considered after flushing with Triton the addition of LNG had no influence and it was quite similar, although the number recovered from the ampulla was ~ 5 times greater than those recovered from the isthmus (Table 1). There were no significant differences between the total number of recovered spermatozoa in the medium with or without the addition of LNG. The observed values were almost five times greater than those observed when we consider the number of spermatozoa at the isthmus or at the ampulla together. Among the samples obtained after flushing with Triton we needed to exclude two samples from each segment because we had technical problems to recover the sperm cells (Tables 1 and 2).

The evaluation of the number of spermatozoa according to the side with or without ovarian follicle and the segment of the tube showed that the number of recovered spermatozoa was similar in the tubal isthmus or ampulla with or without the addition of LNG. Additionally, the number of spermatozoa recovered from the tubes from the side with or without an ovarian follicle did not show significant differences. When we take into account the recovered spermatozoa after

flushing with Triton, also there were no significant differences. Nevertheless, the number of spermatozoa was higher in the ampulla than in the isthmus in both sides and with or without LNG (Table 2).

After the preparation of the slides for acrosomal reaction evaluation some of them showed many epithelial cells and red and white blood cells which make almost impossible to evaluate carefully AR rate and this was the reason that we excluded some evaluations (Tables 3 and 4). The AR rate was also similar in the spermatozoa recovered from the isthmus or from the ampulla when the cells were treated or not with LNG. Also in the medium there were no significant differences between the AR rates when treated with or without LNG (Table 3). When the AR was evaluated in the spermatozoa from the isthmus and from the ampulla with the addition or not of LNG and according to the side with or without ovarian follicle it was noted that there were no significant differences. Additionally, the percentage of AR observed in spermatozoa from the tubal segments and medium from the side with or without an ovarian follicle did not show significant differences (Table 4).

Discussion

We observed that the addition of LNG did not significantly affect the number of recovered spermatozoa neither at the isthmus nor at the ampulla or the number of recovered spermatozoa adhered at the tubal epithelium. When we take into account if the addition of LNG influences the number of recovered spermatozoa, the number of adhered spermatozoa to tubal epithelium, or the

AR rate at the flushing medium in the side in which the ovary presented or not the dominant follicle, also we did not observe significant differences.

However, the main limitation of this study is the design and development of the procedures. The experiments were conducted *in vitro* and although we tested the spermatozoa in the tubal environment it was different than the conditions *in vivo*. The LNG doses used to flushing the tubes are similar to the levels of LNG found in the serum after oral intake as EC and possibly higher than those found in the oviductal fluid. In fact, our group reported previously (18) that the LNG concentration in uterine flushing after oral intake was less than 2% of that found in the serum (16) and probably at tubal lumen the concentrations could be similar. Additionally, our findings can not allow stating that LNG as EC administration *in vivo* could affect the oviductal microenvironment and impair sperm functions. In fact, the best experiment may be that women intake LNG as EC dose previously to removal the tubes and LNG could act, or not, on oviductal progesterone receptors and modify the oviductal microenvironment and its interaction with spermatozoa. Our experiments not mimicked this situation.

Previously, *in vitro* experiments showed that LNG at 10 ng/mL was unable to induce AR [7,17] and also failed to induce significant differences in the number or AR status of spermatozoa recovered from the uterus after administration of 1.5 mg of LNG to sterilized women at different times after coitus [18]. However, to the best of our knowledge, there are no studies in which the effect of LNG was evaluated upon spermatozoa at the site of fertilization. In order to contribute to the understanding of the mechanism of action involved in the contraceptive effect of LNG as an EC our objective was to explore if the drug

could affect the sperm count, the number of adhered spermatozoa to the tubal epithelium, their distribution, and AR in human tubes, *in vitro.*

The methodology used in our study could be not the better one. Initially, we tried to recover spermatozoa from the tubes obtained at the time of sterilization in women who had unprotected coitus and received LNG as EC. However, the number of spermatozoa recovered after perfusion of the tubes was extremely low which can not allow making any further analysis which was also previously observed using the same technique [29]. Consequently we decided to work in vitro, perfused the tubes with a pre-determined number of spermatozoa and evaluated the sperm functions after incubation.

In women, it has been demonstrated that the fertile days of the menstrual cycle are six days including the five days that precede ovulation and the day of ovulation [30]. Recently Noé et al [31] observed that despite the evidence of follicle rupture in women who received LNG as EC before follicle rupture, no pregnancies occurred among those women, suggesting that other mechanism than suppression of ovulation prevents pregnancy when LNG as EC is administered to exposed women.

The sperm migration to the oviducts and the adhesion to the tubal epithelium, a well known mechanism to achieve fertilization, are controlled by the sexual steroids. In rats it was described [27] that E_2 facilitated the sperm migration from the uterus to the oviducts and P modulated this effect. In addition, the interaction between E_2 and P stimulated the sperm adhesion to the tubal epithelium. Although a different sperm reservoir was not observed in human tubes, it was described a functional reservoir and *in vitro* spermatozoa were observed

adhered to the epithelium of the isthmus, albeit in an intermittent manner [32,33]. Ortiz and co-workers [34] observed *in vitro* that after 3 h of incubation the number of spermatozoa adhered/0.1 mm^2 of tubal explants decreased both at the isthmus or the ampulla by the addition of LNG. However, in our study, we used different methodology, because we incubated the tubal segments for 4 h and we flushed the tubal segments with a nonionic surfactant (Triton-X100) and also we recorded the number of spermatozoa obtained at each tubal segment separately. We were unable to observe any influence of LNG on the number of adhered spermatozoa recovered or in their distribution along the Fallopian tubes.

When ovulation is about to take place, spermatozoa experience capacitation and hyperactivation and are able to move forward to the tubal ampulla. Also P modulates many aspects of sperm function and mimics almost entirely the AR-inducing properties of the follicular fluid and the effects of P on sperm function are mediated by receptors located on the plasma membrane [35] defined as nongenomic, since they are rapid and do not involve transcriptional processes [36]. The response is well known to exhibit an absolute dependence on the presence of extracellular bicarbonate [37,38] which is present in intraluminal oviductal fluid in levels higher than in peripheral blood [39].

There are two classes of nongenomic P receptors in the human spermatozoa. One class has an elevated affinity (at nanomolar concentration) and it is specific for P and the other has low affinity (micromolar) and binds also to other hydroxylated P derivatives [36]. When P activates human spermatozoa an increase of the intracellular free calcium is observed and can trigger the process of AR [36,40]. Progestogen like LNG are not able to mimic the effect of P in elevating the

intracellular calcium and seem to be a very weak agonist in nongenomic receptors compared with their potency in affecting the genomic receptor [40].

Regarding the effect of P on the process of AR some studies were carried out with the objective to asses if LNG could affect sperm function like P does. It was observed [8] a direct relationship between AR rate increase and higher concentrations of LNG suggesting that at higher LNG concentrations (200 to 800 ng/mL) the P nongenomic receptors on spermatozoa surface are able to recognize the progestogen molecule and exert its effect. Nevertheless, the studies that used LNG in a similar dose to that observed in serum after oral intake for EC failed to show effect of LNG on AR in spermatozoa *in vitro* [7,17] and also in spermatozoa recovered from the uterus [18].

Conclusions

In conclusion, the hypothesis that LNG in a similar dose than those observed in women's serum after oral intake for EC could affects the number, the adhesion of sperm to tubal epithelium, distribution and AR of spermatozoa at the Fallopian tubes *in vitro* was not confirmed in our study. Our results also failed to show such effect on spermatozoa recovered after exposure to Fallopian tubes even though the LNG doses used *in vitro* are probably higher than those reached within the oviduct *in vivo.*

List of abbreviations

AR: acrosome reaction

CV: coefficient of variation

EC: emergency contraception

E_2: estradiol

FITC-PSA: fluorescein isothiocyanate-labelled *Pisum sativum* lectin

HTF-HEPES: HEPES-buffered modified human tubal fluid medium

LNG: levonorgestrel

P: Progesterone

SEM: standard error of the mean

ZP: zona pellucida

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AH, MEO, HBC and LB participated in the design of the study and in the developing of the research protocol; AH, MVB, FF, NMM, MHRRG and LB conducted the study at the outpatient clinic, surgical theatre and at the laboratory; all the authors contributed equally on written the manuscript and review, revise and have given final approval of the manuscript.

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Table 1. Total number of recovered spermatozoa (x10^3) according to the segment of the Fallopian tube and treatment

	LNG	N	Sperm count (Mean ± SEM)	Range	p-value
Isthmus					0.66
	Yes	13	4.6 ± 1.7	0 - 20	
	No	13	3.8 ± 1.7	0 - 15	
*Isthmus with Triton**					0.23
	Yes	11	0.5 ± 0.2	0 - 2	
	No	11	1.6 ± 0.7	0 - 7	
Ampulla					0.54
	Yes	13	38.9 ± 11.8	0 - 130	
	No	13	33.5 ± 12.1	0 - 140	
*Ampulla with Triton**					0.71
	Yes	11	6.8 ± 2.8	0 - 26	
	No	11	5.2 ± 3.0	0 - 33	
Medium					0.30
	Yes	13	181.9 ± 87.8	0 - 1,200	
	No	13	217.7 ± 85.4	30 - 1,200	

*The spermatozoa from the flushing with Triton were considered adhered to the Fallopian epithelium.

Medium: refers to the medium used in the experiments in which the tubes were immersed during incubation.

Table 2. Total number of recovered spermatozoa ($x10^3$) according to the side with or without ovarian follicle and according to the segment of the tube and treatment

	LNG	N	Sperm count (Mean ± SEM)	Range	p-value
With ovarian follicle					
Isthmus					0.26
	Yes	6	2.5 ± 1.7	0 - 10	
	No	7	7.1 ± 2.6	0 - 15	
*Isthmus with Triton**					0.14
	Yes	5	0.2 ± 0.2	0 - 1	
	No	6	2.3 ± 1.1	0 - 7	
Ampulla					0.40
	Yes	6	51.7 ± 23.7	0 - 130	
	No	7	22.9 ± 10.4	0 - 80	
*Ampulla with Triton**					1.00
	Yes	5	8.8 ± 5.5	0 - 26	
	No	6	3.8 ± 2.0	0 - 12	
Medium					
	Yes	6	67.5 ± 31.2	0 - 190	0.12
	No	7	300.7 ± 152.6	30 - 1200	
Without avarian follicle					
Isthmus					0.07
	Yes	7	6.4 ± 2.8	0 - 20	
	No	6	0.0	0	
*Isthmus with Triton**					1.00
	Yes	6	0.7 ± 0.3	0 - 2	
	No	5	0.8 ± 0.6	0 - 3	
Ampulla					1.00
	Yes	7	27.9 ± 8.7	0 - 65	
	No	6	45.8 ± 23.6	0 - 140	
*Ampulla with Triton**					0.52
	Yes	6	5.2 ± 2.8	0 - 18	
	No	5	6.8 ± 6.6	0 - 33	
Medium					0.68
	Yes	7	280.0 ± 156.4	30 - 1200	
	No	6	120.8 ± 43.3	30 - 285	

*The spermatozoa from the flushing with Triton were considered adhered to the Fallopian epithelium.

Medium: refers to the medium used in the experiments in which the tubes were immersed during incubation.

Table 3. Percentage of spermatozoa with acrosomal reaction according to the segment of the Fallopian tube and treatment

	LNG	N	Acrosome reacted spermatozoa (%) (Mean ± SEM)	p-value
Isthmus				0.57
	Yes	9	15.2 ± 3.3	
	No	9	18.7 ± 2.5	
Ampulla				0.96
	Yes	9	9.1 ± 1.2	
	No	8	9.4 ± 1.9	
Medium				0.28
	Yes	9	5.5 ± 1.9	
	No	9	3.8 ± 2.1	

Medium: refers to the medium used in the experiments in which the tubes were immersed during incubation.

Table 4. Percentage of spermatozoa with acrosomal reaction according to the side with or without ovarian follicle and according to the segment of the Fallopian tube and treatment

	LNG	N	Acrosome reacted spermatozoa (%) (Mean ± SEM)	p-value
With ovarian follicle				
Isthmus				0.15
	Yes	4	7.9 ± 3.2	
	No	5	19.9 ± 3.9	
Ampulla				0.35
	Yes	4	7.5 ± 1.9	
	No	5	11.2 ± 2.5	
Medium				0.77
	Yes	3	5.5 ± 3.1	
	No	5	2.8 ± 3.8	
Without avarian follicle				
Isthmus				0.42
	Yes	5	21.0 ± 3.8	
	No	4	17.1 ± 3.4	
Ampulla				0.40
	Yes	5	10.4 ± 1.5	
	No	3	6.3 ± 2.6	
Medium				0.54
	Yes	6	5.5 ± 2.6	
	No	4	5.1 ± 1.5	

Medium: refers to the medium used in the experiments in which the tubes were immersed during incubation.

3.2. Artigo 2

----- Original Message -----
From: "Fertil Steril" <Fertstert@asrm.org>
To: <bahamond@caism.unicamp.br>
Sent: Thursday, October 13, 2011 4:13 PM
Subject: Fertility and Sterility - Submission Confirmation

Dear Dr Bahamondes,

Your submission entitled "The effect of levonorgestrel as emergency contraception on spermatozoa function: a review." has been received by Fertility and Sterility.

You will be able to check on the progress of your paper by logging on to the Elsevier Editorial System of Fertility and Sterility as an author.
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Thank you for submitting your work to this journal.

Sincerely,

Fertility and Sterility Editorial Office

Running Title: Levonorgestrel and sperm function

The effect of levonorgestrel as emergency contraception on spermatozoa function: a review

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Capsule: Few studies have evaluated the effect of levonorgestrel on sperm function. Oral levonorgestrel used as emergency contraception appears to exert no effect on sperm function; however, results are conflicting.

Structured Abstract

Objective: To review the *in vivo* and *in vitro* effects of levonorgestrel (LNG) as emergency contraception (EC) on sperm function.

Design: Review study.

Setting: A tertiary university center.

Patients: None.

Interventions: None.

Main Outcome Measure(s): *In vitro* and *in vivo* effects of LNG on sperm function.

Results: Seven articles and an abstract containing data on the effects of LNG as EC on sperm function were reviewed. Except for one early study, *in vitro* and *in vivo* studies showed no significant effect of LNG as EC on the number of spermatozoa penetrating the cervical mucus and reaching the uterine cavity, the number of recovered spermatozoa adhered to the fallopian isthmus or ampulla, or the recovered spermatozoa adhered to the human tubal epithelium. Furthermore, no effect was found on sperm-zona pellucida binding capacity or the percentage of spermatozoa with D-mannose receptors when testing physiological doses of LNG. The only significant effect was a dose-dependent impairment of sperm motility.

Conclusions: As EC, LNG apparently does not affect sperm function *in vitro* or *in vivo*. However, further studies should be conducted to evaluate adhesion of spermatozoa to the tubal epithelium and the capacitation process to clarify the mechanism of action of LNG as EC.

Key Words: emergency contraception; levonorgestrel; sperm function.

Introduction

Sperm capacitation occurs during transit though the female genital tract and involves several biochemical changes that lead to an increase in the hyperactivated motility required to penetrate the cumulus cell matrix surrounding the oocyte. Acrosome reaction (AR) involves the fusion and fenestration of the outer acrosomal membrane with the plasma membrane to release the enzymatic content present in the acrosome and takes place on the surface of the zona pellucida (ZP) (1). Progesterone (P) produced by the cumulus (2) induces the influx of calcium into the sperm, producing a physiological stimulus that induces the sperm function related to fertilization such as sperm capacitation, hyperactivation, AR, sperm-ZP interaction and penetration into the oocyte (for review see reference 3). Moreover, sperm chemotaxis to the oocyte is also guided by a P gradient (4) and it is well known that the P present in human follicular fluid (FF) is the steroid responsible for the AR-inducing activity of the FF (5).

The effect of progesterone on spermatozoa does not involve the regulation and transcription of the classical nuclear receptor present in other tissues in the female genital tract, since a non-genomic surface receptor has been described in human spermatozoa (6-8). It has recently been proposed that CatSper, a calcium-sensitive channel, is activated by P. This leads to the assumption that the channel by itself or in association with another protein(s) could be the non-genomic P sperm receptor (9).

Levonorgestrel (LNG) is a synthetic progestin that is widely used as emergency contraception (EC). Currently, the recommended dose for EC consists of two 0.75 mg pills taken 12 hours apart or a single dose of 1.5 mg, taken up to

72 hours following unprotected sexual intercourse (10, 11). The plasma LNG concentration after oral pill intake as EC ranges from 7.9 to 12.3 ng/mL after two oral doses of 0.75 mg 12 hours apart or a single dose of 1.5 mg, respectively (12).

Despite the worldwide use of LNG as EC, its mechanism of action remains a subject of debate. However, it has been well established that its effectiveness increases the closer the drug is administered to the time of coitus, its efficacy being highly dependent on the woman's phase of the menstrual cycle at that time (13,14). The more accepted mechanisms of action reported in the literature include: i) alterations to luteinizing hormone (LH) peak and ovulation, ii) follicular development and luteal interference, iii) an effect on spermatozoa migration and function, and iv) an effect on the fertilization process itself (15-18).

Although synthetic progestins such as LNG have been shown to exert a weak agonistic effect (1% of that observed with P) on non-genomic P receptors in sperm (19), Nikkanen et al (20) observed that local application of LNG in the cauda epididymis of rats impaired the *in vivo* fertilizing potential, suggesting that the drug has a direct effect on spermatogenesis. Since the effect of LNG as EC on sperm function is currently under debate, the objective of this review was to provide an overview of the effect of LNG as EC on several types of sperm function.

Material and methods

We performed a search using the Pubmed, Medline, Embase, ISI Web of Knowledge and ISI Web of Science up to July 31 2011. The key search terms were emergency contraception, levonorgestrel, spermatozoa and sperm functions. Seven articles were found, as well as an abstract from a congress, all of which

included data on the effect of LNG as EC on different types of sperm function. The present review describes the different effects observed.

Results

Sperm migration

In a previous study in which the effects of d-norgestrel were evaluated in women receiving a single 0.4 mg dose orally 3 to 10 hours following sexual intercourse, the effect was found to begin 3 hours after ingestion. At that time, modifications were seen in the cervical mucus that increased over time, reaching a peak 9 hours after ingestion. These effects influenced intracervical penetration by the sperm. In addition, the recovery of spermatozoa present in the intrauterine fluid decreased in proportion to the activity of the drug (21). Another study showed that the administration of LNG to surgically sterilized women in a single 1.5 mg dose given 24 or 48 hours after sexual intercourse or following intrauterine insemination (IUI) showed no effect either on the quality of cervical mucus or on the number of spermatozoa penetrating the cervical mucus and reaching the uterine cavity (14). It was possible to recover viable spermatozoa both from the cervix and the uterine cavity at 36, 48 and 60 hours after coitus (22).

Sperm motility

In one of the aforementioned studies (21), after a single 0.4 mg oral dose of LNG administered 3 to 10 hours after coitus, a pronounced effect was found on the forward motility of sperm recovered from intrauterine fluid at 9 and 10

hours after ingestion. The number of motile spermatozoa in the intrauterine fluid diminished as the effect of the drug increased. This observation may be related to the pronounced alkalinization of uterine pH that was found from 5 hours onwards (21). Additionally, Yeung et al. (23) showed that when human spermatozoa were treated *in vitro* with LNG at doses of 1 ng/mL, 10 ng/mL or 100 ng/mL for 3 hours, a dose-dependent impairment of motility was found. When 10 or 100 ng/mL doses of LNG were used, curvilinear velocity (VCL) and straight-line velocity (VSL) of the treated spermatozoa were found to decrease. Average path velocity (VAP) and linearity (VSL/VCL) were affected only with the 100-ng/mL dose of LNG.

Tubal transport

In one study (24), human spermatozoa were incubated with ampulla and isthmus explants in a culture medium with or without 7.8 ng/mL of LNG. LNG significantly decreased the number of spermatozoa adhered to both the isthmus and ampulla explants. This study gives strength to the hypothesis that LNG as EC could affect the interaction between spermatozoa and the endosalpinx and also some forms of sperm function.

With the technical limitations involved in obtaining sperm from the human reproductive tract, an *in vitro* approach was recently developed in this laboratory. Human fallopian tubes removed during sterilization were perfused with a suspension of $1x10^6$ motile spermatozoa in a culture medium either containing 7.8 ng/mL of LNG or not, and further incubated at 37ºC in the presence or absence of LNG (25). In the LNG and control groups, respectively, the numbers of motile spermatozoa ($x10^3$) found at the isthmus were 4.6 and 3.8, while 38.9

and 33.5 were found at the ampulla. The number of recovered spermatozoa ($x10^3$) adhered (after treatment with Triton-X100) to the isthmus was 0.5 and 1.6, while the number adhered to the ampulla was 6.8 and 5.2, in the LNG and control groups, respectively. These results show that the addition of LNG did not significantly affect the number of motile spermatozoa recovered either at the isthmus or at the ampulla, or the number of recovered spermatozoa adhered to the human tubal epithelium. Furthermore, no significant differences were found even when the experiment was controlled by taking the side on which the woman ovulated into account.

Acrosome reaction (AR)

In one study, Bahamondes *et al.,* (26) exposed capacitated human spermatozoa *in vitro* to three different concentrations of LNG ranging from 200 to 800 ng/mL. After 30 minutes of incubation all the LNG concentrations were found to have induced a significant increase in the percentage of acrosome-reacted spermatozoa compared to controls and this effect was dose-dependent. The maximum effect was observed with human FF in which P concentration was 124 µg/mL. It is expected that during transit through the oviduct, most of spermatozoa have intact acrosomes and only a few undergo spontaneous AR (1). Considering that fertilizing spermatozoa undergo AR on the oocyte surface (27), it is suspected that the premature induction of the AR with specific substances such as LNG may decrease the cohort of spermatozoa with fertility potential.

To test the effect of different doses of LNG on the AR rate (28), spermatozoa were incubated under capacitating conditions and then exposed to

either 1,000 or 10,000 ng/mL of LNG (comparable to the dose delivered by the levonorgestrel-releasing intrauterine system [LNG-IUS]) or control medium. Results showed that both 1,000 and 10,000 ng/mL of LNG significantly increased the proportion of acrosome-reacted cells in comparison to controls. Therefore, it is reasonable to speculate that higher LNG concentrations may exert an agonistic effect, probably by binding to the non-genomic P receptor, stimulating calcium uptake and triggering the occurrence of the AR (19).

Nevertheless, when human spermatozoa were treated *in vitro* with LNG concentrations of 1 ng/mL, 10 ng/mL or 100 ng/mL (23, 29), representing lower, similar and higher levels compared to those observed in the serum of women following ingestion of LNG as EC, no significant differences were found in the AR rate. In order to simulate the *in vivo* conditions, one of the studies mentioned above (25) also evaluated AR in spermatozoa incubated in human tubes with or without 7.8 ng/mL of LNG and then recovered after perfusion from the isthmus or from the ampulla. The percentages of AR found in the spermatozoa recovered from the isthmus were 15.2% and 18.7%, while the percentages recovered from the ampulla were 9.1% and 9.4% after incubation with and without LNG, respectively. No significant differences were found between the cells incubated with LNG and those incubated without LNG, and motility was normal in all the spermatozoa recovered.

The effect of LNG was also studied *in vivo* when administered to surgically sterilized women as EC in a single 1.5 mg dose given 12 or 36 hours after sexual intercourse or 24 hours after IUI (22). AR status was evaluated in spermatozoa recovered from the cervical mucus and from the uterine cavity after uterine flushing performed 24 or 48 hours after EC pill intake. The

percentage of AR in the cervical mucus ranged from 9.3% to 10.2% in those spermatozoa obtained from women treated with placebo and from 8.0% to 12.5% in the women treated with LNG. The AR rate observed in the spermatozoa obtained after uterine flushing ranged from 6.2% to 12.7% and 7.8% to 13.0% in the placebo- and LNG-treated groups, respectively. No statistically significant differences were found between the AR rates in the LNG and placebo groups at the different times of LNG exposure after sexual intercourse or after IUI.

Zona pellucida (ZP) binding

Using the hemizona assay, Yeung *et al.* (23) investigated whether different concentrations of LNG would exert any effect on the ZP binding capacity of human spermatozoa. These investigators reported that at concentrations of 1 and 10ng/mL, LNG did not affect sperm-ZP binding capacity; however, when 100 ng/mL was used, a statistically significant decrease of 3% was found in the number of bound spermatozoa compared to control hemizona in culture medium.

D-mannose mediates sperm-egg interaction, since specific carbohydrate binding sites on the sperm surface recognize mannose glycol-conjugates present at the ZP (30). There is some evidence that the P receptor and the D-mannose binding site (or ZP receptor) are topographically related on the sperm surface (31). Taking these two hypotheses into account, another study treated spermatozoa with 1, 10 or 100 ng/mL of LNG and exposed them to whole oocytes instead of hemizona. D-mannose binding site expression was also evaluated as a zona affinity parameter (32). Results showed that the exposure of human spermatozoa to LNG *in vitro* did not affect the number of spermatozoa

tightly bound to the ZP. When exposed to the different concentrations of LNG, the number of bound spermatozoa was similar to that of spermatozoa incubated with the oocytes in culture medium. The effect of LNG on the development of the mouse embryo was also analyzed, with no difference being found between those exposed and those not exposed to LNG. In accordance, no effect on the expression of D-mannose- binding sites on sperm surface was also observed. Both studies (23, 32) support the idea that the effect of LNG as EC cannot be explained by an effect on the mechanism involved on the sperm–ZP interaction or on the fertilization process itself.

To test the effect of different doses of LNG on the detection of D-mannose binding sites, capacitated spermatozoa were exposed for 30 minutes to 1,000 or 10,000 ng/mL of LNG (a dose comparable to that delivered by the LNG-IUS) or control medium (28). Results indicated that at both concentrations LNG significantly increased the percentage of spermatozoa with D-mannose receptors localized in pattern III compared to controls and that this increase was dose-dependent. Pattern II was not modified since the values found in the treated spermatozoa remained similar to those found in the controls. Following staining with dual-color labeling, this effect was found to be in accordance with the improvement observed in the number of acrosome-reacted spermatozoa. The zona binding site in acrosome-intact spermatozoa was located throughout the whole acrosome, while in sperm undergoing AR or in sperm that have just reacted the mannose receptor was distributed as a band around the equatorial segment. These findings suggest that there is an association between acrosome status and the position of the mannose-binding site, suggesting that by

increasing the number of acrosome-reacted pattern III cells, high concentrations of LNG may decrease sperm fertilizing capacity.

Conclusions

At a single dose of 1.5 mg taken up to 72 hours after unprotected sexual intercourse, LNG is the most widely used and best accepted method of EC despite the recent introduction of new EC pills such as ulipristal acetate (33, 34) in the USA and several European countries. Despite the large body of evidence concerning the effectiveness of LNG as EC taken up to 72 hours following unprotected sexual intercourse, there remain several unresolved questions regarding its mechanism of action, principally with respect to its effect on sperm function and sperm penetration in the female reproductive tract (11).

A recent declaration from the International Federation of Obstetrics and Gynecology and the International Consortium for Emergency Contraception (35) on the mechanism of action of LNG pills as EC on sperm function stated: "*Contradictory results exist regarding whether LNG taken post-coitally and in doses used for EC affects sperm function, and according to the results, the mechanism of action is still uncertain and warrants further studies*". The present review shows that few studies have focused on the effect of LNG as EC on sperm function despite the fact that this mechanism of action is plausible. Furthermore, the different studies used different evaluation methods and results were in many cases contradictory. Table 1 presents a summary of the different studies.

Many years ago, Wilcox *et al.* (36) conducted a seminal study showing that women are only fertile for six days of the menstrual cycle: the five days that

precede ovulation and the day of ovulation itself. During sexual intercourse spermatozoa are deposited in the vagina and remain in the cervical crypts for many hours or days before ascending to the Fallopian tubes (37). Consequently, in women, it is reasonable to speculate that these spermatozoa are affected when LNG pills are taken as EC since the spermatozoa would be exposed to unknown concentrations of LNG for hours or days depending on the interval between sexual intercourse and EC pill intake. This may affect their fertilizing capacity.

Several publications on the mechanism of action of LNG as EC have concluded that the pills interfere both with sperm penetration and motility and with the quality of cervical mucus (13, 14). This statement was based mainly on a previous study conducted by Kesseru *et al.* (21) and published more than 30 years ago. That study showed that following a single dose of d-norgestrel taken after sexual intercourse there was a reduction in the number of spermatozoa recovered from the uterine cavity, as well as alterations in the pH of the uterine fluid, which provoked sperm immobilization and an increase in cervical mucus viscosity. Although the study was much cited, the methods used were not the same as those currently used. In short, sperm fertilizing capacity can be assessed by using other sperm function tests such as the occurrence of spontaneous and induced AR, ZP-binding capacity, detection of D-mannose binding sites or interaction with the tubal epithelium, which are considered more appropriate today. This is probably the principal reason why the results from many recent studies (22, 23, 26, 28, 29, 32) failed to confirm those early findings.

At the time of sexual intercourse, human spermatozoa are deposited in the vagina and very quickly enter into contact with the cervical mucus and

penetrate the cervix. The cervical mucus separates those spermatozoa with abnormal morphology and motility so that only a minority of the spermatozoa is able to penetrate the cervix (37). Although LNG has been reported to affect cervical mucus and sperm motility, resulting in impaired sperm penetration, these effects were not confirmed in recent studies conducted by different groups (21-23).

The muscular contractions of the uterus exert a favorable effect on sperm penetration thorough the uterine cavity. Thousands of spermatozoa migrate through the uterine-tubal junction to reach the fallopian tubes where the spermatozoa are stored maybe in a reservoir or at least maintained in fertile form through interaction with the tubal epithelium. The interaction between spermatozoa and the endosalpinx offers some protection to spermatozoa and *in vitro* studies have shown that sperm motility and fertility were longer lasting when spermatozoa were incubated with tubal epithelium (38). Close to the time of ovulation, the spermatozoa undergo capacitation and hyperactivation to enable them to continue their journey towards the ampulla (37).

When spermatozoa were incubated (24) with human tubal explants from the ampulla and the isthmus in culture medium with or without LNG, it was found that LNG diminished the interaction of spermatozoa with the tubal epithelium. However, in an *in vitro* experiment with similar characteristics (25) in which our group worked with both human tube segments perfused and incubated with a suspension of spermatozoa in culture medium with or without LNG, no effect was found with respect to the number of motile spermatozoa recovered. This discrepancy may be a consequence of different experimental approaches. Ortiz *et al.* (24) assessed the number of spermatozoa adhered/0.1 mm^2 of tubal

explant; however, in the work carried out by our group (25) the tubal segments were flushed with a nonionic surfactant (Triton-X100) and the numbers of motile spermatozoa obtained after flushing each segment separately were recorded.

Progesterone mimics the AR-inducing properties of the FF almost perfectly and its effects on sperm function are mediated by nongenomic receptors located in the plasma membrane (3,8). There are two classes of nongenomic P receptors in human spermatozoa: one with a high affinity (at nanomolar concentrations) that demonstrates high structural specificity for the steroid and is unable to interact with P analogs or antiprogestins, while the other has low affinity (at micromolar concentrations) and also binds to other hydroxylated P derivatives (8, 39).

It has recently been reported that nanomolar concentrations of P stimulate a pH-dependent Ca(2+) channel called CatSper, which is localized in the human sperm flagellum (40). That experiment suggested that CatSper, or a directly associated protein, functions as a non-genomic P receptor. Since synthetic progestins such as LNG have already been shown to be weak agonists of the P sperm receptor, provoking an increase in Ca(2+) leading to AR (19), the affinity of progestins for this P receptor should be investigated.

According to the results observed in the literature, when LNG is used as EC, it is probable that the drug does not reach sufficient serum concentrations for it to be recognized by the sperm P receptors and exerts its agonist effect. Positive results were only observed when the dose of LNG used in the experiments was much higher (comparable to that found with the use of the LNG-IUS) and not at the doses proposed for use as EC (26, 28). At higher concentrations, LNG may also

bind to the sperm P receptor, stimulating calcium uptake and the occurrence of the AR (19). Therefore, with respect to the effect of LNG on AR it is possible to conclude that LNG, when used at similar doses to those observed in human serum following ingestion does not affect the rate of AR and this is probably not one of its mechanisms of action. It is important to take into account that the plasma levels of LNG following ingestion as EC range from 8.4 to 12.5 ng/mL after two doses of 0.75 mg 12 hours apart or a single dose of 1.5 mg, respectively (12). These doses are lower than many of those evaluated in the various studies.

Yeung *et al*. (23) reported that LNG *in vitro* had no effect on sperm AR; however, it affected sperm motility and spermatozoa–oocyte fusion, this effect being more evident at high concentrations of the steroid (100 ng/mL). Despite a decrease observed in the number of spermatozoa tightly bound to human ZP in the LNG group, Munuce *et al.* (32) found no statistically significant differences between the number of bound spermatozoa exposed to different concentrations of LNG and the number of spermatozoa incubated in culture medium. Both studies (23, 32) support the idea that the effect of LNG as EC cannot be explained by the decrease in the number of spermatozoa with affinity for the ZP.

Recently Noé *et al.* (41) observed that despite the evidence of ovarian follicle rupture in women who received LNG as EC prior to follicle rupture, no pregnancies occurred in these women, suggesting that mechanisms other than ovulation suppression prevent pregnancy when LNG is administered as EC to exposed women. In conclusion, our recommendation is that, after LNG intake or following *in vitro* exposure to LNG in the fallopian tubes, the different types of sperm function such as the adhesion of spermatozoa to the tubal epithelium and

the events that occur during the capacitation process, among others, need to be studied more extensively in order to clarify the mechanism of action of LNG as EC. Further studies should be conducted to evaluate the viscosity and other properties of cervical mucus and, consequently, sperm penetration into the uterine cavity following the ingestion of LNG as EC.

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Table 1. Summary of research articles evaluating the effects of LNG on sperm function

Author/year	Dose	Study	Sperm function	Effect
Kesseru et al.(1974)	0.4mg single oral dose, 3-10 h after sexual intercourse	in vivo	sperm penetration sperm motility	yes yes
Yeung et al. (2000)	1, 10, 100ng/mL	in vitro	sperm motility AR ZP binding	yes(10,100ng/mL) no yes (100ng/mL)
Bahamondes et al.(2003)	200, 400, 800ng/mL	in vitro	AR	yes
Brito et al. (2005)	1, 10, 100ng/mL	in vitro	AR	no
Munuce et al. (2005)	1, 10, 100ng/mL	in vitro	ZP binding α-Dmannose binding sites	no no
Munuce et al. (2006)	1,000 and 10,000ng/mL	In vitro	AR α-Dmannose binding sites	yes yes
do Nascimento et al. (2007)	1.5mg single oral dose 24-48 h after sexual intercourse	in vivo	Sperm penetration AR	no no
Ortiz et al. (2009)	7.8ng/mL	in vitro	adhered sperm	yes
Hermanny et al. (2011)	7.8ng/mL	in vitro	sperm motility AR adhered sperm	no no no

4. Discussão

Os resultados deste trabalho mostraram que o LNG, na concentração utilizada de 7,8ng/ml (25nM), não afetou significativamente o número de espermatozoides móveis recuperados do istmo e da ampola. O número de espermatozoides aderidos ao epitélio tubário e a taxa de RA também não sofreram influência do progestágeno. Quando se considerou que o LNG poderia afetar diferentemente os resultados, avaliando a tuba uterina do lado em que o ovário apresentava folículo dominante ou não, também não foram observadas diferenças significativas.

A técnica utilizada neste estudo talvez não seja a ideal. Inicialmente tentou-se recuperar os espermatozoides lavando os segmentos de tubas uterinas retiradas de mulheres que haviam recebido LNG após um coito desprotegido. Porém, o número de espermatozoides recuperados foi muito pequeno e não possibilitou uma avaliação adequada das características funcionais, o que foi também observado por outros pesquisadores (29). Esta limitação provocou a adoção de uma nova técnica, *in vitro*.

Após o coito, as contrações musculares do útero favorecem a penetração dos espermatozoides na cavidade uterina, e em seguida migram atravessando a junção útero-tubárica até chegarem às tubas uterinas, onde permanecem à espera da ovulação. Nas tubas, a capacidade fertilizante dos espermatozoides é mantida pela interação com o epitélio tubário. Foi mostrado que, *in vitro*, quando espermatozoides são incubados com tecido tubário, a motilidade e a fertilidade espermática são mantidas por mais tempo (25). Com a aproximação da ovulação, os espermatozoides sofrem capacitação e migram em direção à ampola (13).

Neste trabalho, os resultados encontrados mostraram que o LNG, na concentração utilizada, não afetou o número de espermatozoides móveis recuperados tanto do istmo como da ampola, e também não afetou o número de espermatozoides aderidos ao epitélio de cada segmento tubário. Resultado diferente foi observado por Ortiz e colaboradores (28) quando incubaram espermatozoides com explantes de istmo e ampola em meio de cultura com e sem LNG, e verificaram que a interação dos espermatozoides com o epitélio tubário foi menor na presença do LNG. A discrepância entre os resultados destes dois trabalhos talvez possa ser explicada pela utilização de metodologias diferentes. Ortiz e colaboradores (28) consideraram o número de espermatozoides aderidos/0,1mm^2 de explante tubário enquanto que, neste trabalho, os segmentos tubários foram perfundidos com um surfactante não iônico (Triton-X100) e foi considerado o número de espermatozoides recuperados de cada segmento separadamente.

A capacitação espermática é um evento que ocorre durante a passagem do espermatozoide pelo trato genital feminino e envolve mudanças bioquímicas que levam a um aumento na motilidade hiperativada, necessária para que o espermatozoide ultrapasse o *cumulus* que envolve o oócito e a RA. A RA acontece na superfície da ZP e, através da fusão e fenestração da membrana acrossomal externa com a membrana plasmática do espermatozoide, o conteúdo enzimático presente no acrossoma é liberado (20). A P produzida pelo *cumulus* (30) provoca um influxo de cálcio extracelular para dentro do espermatozoide, e funciona como estímulo fisiológico na indução de funções espermáticas relacionadas com a fertilização que incluem capacitação, RA, hiperativação, interação com a ZP e penetração do oócito (31).

O FF tem a capacidade de induzir a RA, e a P presente no FF é o esteróide responsável por esta ação (32). A P reproduz quase que totalmente as propriedades do FF como indutora de RA e seus efeitos na função espermática são mediados por receptores não genômicos localizados na membrana plasmática dos espermatozoides (31,33). No espermatozoide humano foram descritas duas classes de receptores não genômicos: uma de elevada afinidade, sensível a concentrações nanomolares, e de alta especificidade estrutural, incapaz de interagir com análogos de P, e outra classe, de baixa afinidade, sensível a concentrações micromolares e capaz de se ligar também a derivados da P (33; 34). Recentemente foi proposto que o Catsper, um canal sensível ao cálcio e que é ativado por concentrações nanomolares de P, poderia ser ele próprio ou associado a outras proteínas, o receptor espermático não genômico (35).

Os resultados encontrados neste trabalho mostraram que o LNG, em concentração semelhante aos níveis plasmáticos encontrados após a ingestão para AE, não afetou a taxa de RA observada nos espermatozoides recuperados do istmo e da ampola. Este resultado está de acordo com aqueles observados por outros pesquisadores, *in vitro*, ao incubarem espermatozoides com LNG em concentrações semelhantes às observadas após o uso na AE (18; 22), e *in vivo*, após analisarem a taxa de RA em espermatozoides recuperados do muco cervical e do útero de mulheres cirurgicamente estéreis que haviam recebido LNG como AE (23).

Foi mostrado que progestágenos sintéticos como o LNG são agonistas fracos dos receptores espermáticos de P e incapazes de promover um aumento nos níveis de Ca que induza à RA (36). A concentração plasmática de LNG após o tratamento para AE varia de 7,9 a 12,3ng/ml (5). Os trabalhos que abordaram o efeito do LNG nas funções espermáticas mostraram que, quando usado na AE, o LNG provavelmente não atinge concentração plasmática suficiente para ser reconhecido pelos receptores de P e não exerce efeito na célula espermática. Resultados positivos só foram observados quando a dose de LNG utilizada nos experimentos foi muito maior que a preconizada na AE (17; 19). Em altas concentrações, parece que o LNG consegue se ligar aos receptores de P, estimulando o aumento de Ca intracelular e RA (36). Portanto, considerando o efeito do LNG na RA pode-se concluir que, quando usado em doses similares às observadas nos níveis plasmáticos após a ingestão para AE, o LNG não afeta a taxa de RA e, portanto, este não é um dos seus mecanismos de ação.

Os trabalhos encontrados na literatura que abordam o efeito do LNG, quando usado na AE, sobre os diferentes aspectos da função espermática, muitas vezes apresentaram resultados contraditórios. Apesar de Kesseru e colaboradores (15) terem descrito que o LNG alterou o muco cervical e a motilidade dos espermatozoides, comprometendo a penetração espermática, este efeito não foi confirmado por estudos mais recentes, realizados por diferentes grupos (18; 23). Yeung e colaboradores (18) observaram que, *in vitro*, o LNG, na concentração de 100ng/ml, portanto maior que a observada nos níveis séricos após a ingestão de pílulas para AE, apesar de não afetar a RA, afetou a fusão do espermatozoide com o oócito. Usando esta mesma concentração de LNG, porém com outra metodologia, Munuce e colaboradores (37) não encontraram diferença entre o número de espermatozoides ligados a ZP após a incubação com e sem LNG. Entretanto, na concentração de 10ng/ml, ambos os grupos não observaram influência do LNG na capacidade dos espermatozoides se ligarem a ZP, sugerindo que o efeito do LNG como AE não poderia ser explicado por um comprometimento na interação entre espermatozoide e ZP ou no próprio processo de fertilização.

O mecanismo de ação do LNG como AE não está totalmente esclarecido e existem poucos estudos que analisam seu efeito nas funções espermáticas, embora este seja um possível mecanismo de ação, com resultados muitas vezes contraditórios. Portanto, acredita-se que mais estudos poderiam ser realizados abordando possíveis alterações nas diferentes funções espermáticas, tais como aderência ao epitélio tubário e eventos que ocorrem durante o

processo de capacitação, após o uso do LNG com AE ou *in vitro*, expondo as tubas uterinas ao progestágeno. Também o efeito do LNG sobre a viscosidade e outras características do muco cervical, ou seja, sobre a penetração do espermatozoide na cavidade uterina, poderia ser mais intensamente estudada para tentar contribuir com o mecanismo de ação do LNG na AE.

5. Conclusões

- O LNG não afetou o número de espermatozoides móveis recuperados tanto do istmo como da ampola tubária.
- O número de espermatozoides aderidos tanto ao istmo como à ampola tubária foi semelhante após incubação com e sem LNG.
- O LNG não afetou a taxa de reação acrossomal observada nos espermatozoides recuperados do istmo e da ampola tubária.
- A presença do folículo dominante não influenciou os resultados.
- De acordo com a literatura, o possível efeito do LNG como AE sobre diferentes funções espermáticas ainda é bastante contraditório.

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7. Anexos

7.1. Anexo 1 – Tabela

Distribuição percentual dos espermatozódes recuperados

Segmento	LNG no meio	N	Espermatozoides recuperados (%)
Istmo	Sim	13	2,00
	Não	13	1,47
Istmo com Triton	Sim	11	0,17
	Não	11	0,53
Ampola	Sim	13	16,78
	Não	13	12,83
Ampola com Triton	Sim	11	2,50
	Não	11	1,68
Meio	Sim	13	78,60
	Não	13	83,48